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AD NUMBER
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AUTHORITY
U.S. Army Medical Research and Materiel Command ltr., dtd March 21, 2001.

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AD_____

Award Number: DAMD17-97-1-7103

TITLE: The Use of a Human Breast Tumor Progression Series and a
3-D Culture Model to Determine if Nuclear Structure Could
Provide a Molecular and Therapeutic Marker

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REPORT DATE: January 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
               Fort Detrick, Maryland   21702-5012

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Patricia Madsen   12/26/60

REPORT DOCUMENTATION PAGE

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1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE January 2000	3. REPORT TYPE AND DATES COVERED Final (30 Sep 97 - 31 Dec 99)

4. TITLE AND SUBTITLE
The Use of a Human Breast Tumor Progression Series and a 3-D Culture Model to Determine if Nuclear Structure Could Provide a Molecular and Therapeutic Marker

5. FUNDING NUMBERS
DAMD17-97-1-7103

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
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12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Alteration in nuclear structure is a hallmark of cancer cells. Nuclear structure partly relies on the supramolecular organization of proteins called nuclear matrix (NM) proteins. By investigating the appearance or disappearance of NM proteins on 2D gels prepared from various stages in a human breast progression series, we have identified potential tumor suppressor or promoter candidates. In addition, using a model of human mammary epithelial cell (HMEC) differentiation and tumorigenesis, we have demonstrated that the NM protein NuMA progressively redistributes within nuclei only during acinar differentiation. Interestingly, cell membrane and NuMA organizations influence each other in acinar cells: Alteration of cell-cell and cell membrane-extracellular matrix (ECM) interactions changes NuMA distribution; conversely, alteration of the supramolecular organization of NuMA abolishes cell membrane-ECM interaction and also induces changes in chromatin structure and increased sensitivity to apoptosis. This indicates that NuMA is a regulator of HMEC differentiation. Moreover, we have developed an imaging algorithm that permits quantitative measurements of differences in NuMA distribution and discrimination between non-malignant and malignant cells even in conditions where the eye cannot separate the staining patterns (e.g., in non-differentiated proliferating cells). These results suggest that the study of NM proteins offers new potentials for anticancer strategies.

14. SUBJECT TERMS Breast Cancer , nuclear organization, extracellular matrix signaling	15. NUMBER OF PAGES 56
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Limited

NSN 7540-01-280-5500

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_____   1/29/2000
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Sophie A. Lelièvre.

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Abbreviations:
3D-rBM: Three-dimensional reconstituted basement membrane; BM: Basement membrane;
ECM: Extracellular matrix; HMECs: Human mammary epithelial cells; NM: Nuclear matrix;
NMPs: Nuclear matrix-associated proteins.

INTRODUCTION.

The purpose of the proposed research was to identify nuclear structural proteins that play a role in the regulation of human mammary epithelial cell (HMEC) phenotype. Structural nuclear proteins are defined as proteins that remain with the cell and nuclear scaffolds following removal of soluble proteins and DNA. It has been proposed that these proteins play a role in the regulation of cell phenotype by organizing nuclear (and to some extent cytoplasmic) function.

This project relied on the use of a human progression series HMT-3522 developed more than a decade ago by Briand and colleagues in Denmark. More recently these cells had been used to develop an *in vitro* model of differentiation and tumorigenesis (Petersen et al., 1992) in the laboratory of Dr. Mina J. Bissell at the Lawrence Berkeley National Laboratory (Berkeley, CA). In this model cells are grown in three-dimension in the presence of a reconstituted extracellular matrix (ECM) enriched for basement membrane components (3D rBM culture). When grown in 3D-rBM, the non-malignant S1 cells of the HMT-3522 series undergo differentiation and form normal tissue-like glandular structures (acini) in which growth-arrested cells vectorially secrete products in a central lumen. Whereas, the tumorigenic T4-2 cells of the HMT-3522 progression series form continuously growing and disorganized tumor-like nodules when cultivated in the same conditions. An intermediate stage in the progression series (S2 cells) as been classified as a pre-malignant stage since these cells are non-tumorigenic but fail to undergo acinar morphogenesis, and instead form continuously growing cell clusters in 3D rBM culture.

RESULTS.

Using the HMT-3522 cell model I have shown during the first year of funding that formation of normal breast tissue structures (acini) was accompanied by the redistribution of several nuclear structural proteins and that this nuclear reorganization was in fact directing the HMEC phenotype. Notably, I have shown that the nuclear structural protein NuMA is an essential mediator of ECM-induced acinar morphogenesis, and that its nuclear distribution in acinar cells is linked to the deposition of a continuous basement membrane around the acinus. Moreover, in collaboration with Dr. R. Getzenberg (Pittsburgh, PA), we have identified three potential tumor suppressor candidates and three potential tumor promoter candidates by comparing 2D gels of nuclear matrix associated proteins (NMPs) prepared from the various stages of the HMT-3522 progression series. (See first annual report, 1998)

In this report for the second and final year of support of this project, I present confirmation of previous results as well as new results regarding the NMPs (collaboration with Dr. Getzenberg). Then, I emphasize the work done with the NuMA project which clarifies the role played by this protein in maintaining acinar differentiation and identifies part of the molecular mechanisms by which NuMA exerts its function. I also present preliminary data on the utilization of NuMA distribution as a marker of HMEC phenotype.

1) <u>Objective 1 and 2 of the SOW</u>: *To identify nuclear matrix associated proteins (NMPs) that may play a role in tumorigenesis, using 2D gel analysis of NMPs in the HMT-3522 progression series in collaboration with Dr. R. Getzenberg.* The human HMT-3522 breast epithelial progression series includes S1 cells that were isolated from reduction mammoplasty of a fibrocystic disease, and became immortalized in culture (Briand et al., 1987). Pre-malignant S2 cells, derived from S1 cells, were obtained by removing epidermal growth factor from the culture medium. Continuous culture of S2 cells on a plastic surface (2D monolayer) ultimately gave rise to tumorigenic cells. Epithelial T4-2 tumor cells were obtained from tumors given by S2 cells at passage 238. T4 tumor cells were further propagated as 2D monolayer (Briand et al., 1996).

In this part of the research project, we were looking for proteins the expression of which was either lost or activated in malignant T4-2 cells compared to pre-malignant S2 cells, as well as proteins the expression of which was lost or activated in pre-malignant S2 compared to non-malignant S1 cells. When grown in 3D rBM S1 cells form differentiated acini, S2 cells form cyst-like structures, and T4-2 cells form tumor-like nodules; hence these cultures recapitulate situations comparable to *in vivo*. 2D gels were performed with cells grown both as a 2D monolayer and in 3D rBM. The hypothesis was here that the combined use of the progression series and the 3D rBM culture system would unable us to narrow down the possibilities in order to identify meaningful changes in the NMPs pattern on 2D gels. In the previous report, I had indicated that we had identified three proteins that disappeared from S1 to S2 cells and therefore were tumor suppressor candidates, and three proteins that appeared from S2 to T4-2 cells and therefore were tumor promoter candidates. Most of those results have been now confirmed. Moreover, we have also identified a few other proteins that may play a role in the progression of S1 cells towards the loss of acinar differentiation (very early steps in tumor progression) by analyzing S1-117 and S1-178 cells that constitute late passages of S1 cells. More

specifically S1-178 cells still form acinar-like structures in 3D rBM, but the cell clusters are bigger (indicating a delay in the process of growth-arrest) and they show altered cell and nuclear organization. Each of these analyses was very time consuming and took several months because of the number of gels to be analyzed. Further investigation will include the sequencing of these proteins and the development of antibodies. These results could lead to the use of the identified NMPs as markers of tumor progression and targets for anti-cancer treatment (Konety and Getzenberg; 1999).

2) <u>Objective 3</u>: *Study of the role of NuMA distribution in differentiation and tumorigenesis of HMECs.* This objective is based on the hypothesis that nuclear structural proteins may participate in the regulation of cell differentiation by changing their localization within the nucleus. The nuclear mitotic apparatus protein, NuMA, which plays a role in mitotic spindle organization and nucleus re-formation, is highly abundant in interphase in both acinar and malignant HMECs, but its localization is different in these two situations. Therefore the study of NuMA had been proposed in the original research application. The first report presented results demonstrating that acinar morphogenesis was accompanied by the progressive re-distribution of NuMA within nuclei. Other nuclear structural proteins, the function of which is already known (e.g., Rb and splicing factors SRm160) also altered their distribution upon completion of acinar morphogenesis.

When NuMA organization was altered in acinar cells (using anti-NuMA antibody following short permeabilization of living cells), differentiation was lost, as shown that the degradation of the basement membrane surrounding the acinus, and chromatin structure was modified, as exemplified by alteration in the distribution pattern of acetylated histone H4. These data demonstrated for the first time that ECM-induced formation of tissue structure is accompanied by the re-organization of the cell nucleus, and that nuclear organization, itself, directs cell phenotype. These results are now published in Proc. Natl. Acad. Sci. (Lelièvre et al., 1998; see appendix). The first report also included data indicating that, when tumor T4-2 cells are induced to revert and form acini by treating them with either anti-beta-1 integrin of anti-EGFR blocking antibodies (Weaver et al.,1997; Wang et al., 1998), NuMA distribution becomes similar to that observed in S1 acinar cells, thus confirming the importance of NuMA re-localization in acinar differentiation (Lelièvre et al., in manuscript form). Finally, other results demonstrated that NuMA organization in acinar cells is dependent on cell membrane organization. Treatments that altered cell-cell and cell-ECM interactions (short EDTA treatment) induced NuMA proteolysis (40 kD lost) and a modification of its organization. Such phenomenon did not seem to be accompanied by an alteration of phosphorylation and it could not be reproduced by altering cytoskeletal arrangement (Lelièvre et al., in manuscript).

a) <u>Role played by NuMA in the regulation of HMEC phenotype.</u>

Outside-in signaling:
During the second year of funding, I have confirmed that there is a specific relationship between NuMA distribution into enlarged and peripheral foci in the nucleus of acinar cell and the presence of an organized cell membrane by treating revertant tumor cells (RT4) and tumor cells (T4-2) grown in 3D rBM (day 10) with EDTA. EDTA-induced alteration of cell-cell and cell-ECM interactions in revertant cells arranged into acini triggered changes in NuMA organization similar to that observed in S1 cells (i.e., diffusion of NuMA throughout the nucleus or collapse in the center of the cell nucleus, as seen in NM preparations). Tumor cells did not show such a reorganization. However, in all cases NuMA was proteolized (Lelièvre et al., in manuscript form). This suggests that the cell

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membrane-triggered biochemical pathway that directs NuMA proteolysis is intact in tumor cells; the fact that NuMA distribution is only altered in acinar cells probably reflects specific binding characteristics for this protein acquired upon acinar differentiation.

Inside-out signaling:
During the second year of funding I have confirmed that the distribution of NuMA in acinar cells maintains the differentiated phenotype. I have analyzed the effect of disrupting NuMA organization using anti-NuMA antibody in revertant RT4-2 cells that have formed acini, and also in S1 cells transfected with EGFR and tumor cells that do not form acini and keep proliferating in 3D rBM. Before treatment, RT4 cells showed NuMA distributed into enlarged and peripheral foci and the presence of an intact basement membrane around the acini, as illustrated by collagen IV staining. S1-EGFR cells showed NuMA diffusely distributed and the presence of an intact basement membrane around the cell clusters. T4-2 cells showed NuMA diffusely distributed and either little deposition or aberrant deposition of basement membrane. Only cells that presented both the distribution of NuMA into enlarged peripheral foci and an intact basement membrane were affected by the treatment with anti-NuMA antibody. In this case, NuMA became diffusely distributed and the basement membrane was degraded upon the activation of metalloproteinases (Figure 1; Lelièvre et al., in manuscript form).

Studies performed on S1 acinar cells have shown that the loss of acinar differentiation following antibody-induced NuMA disorganization was accompanied by an increased apoptosis sensitivity. Treatment of "anti-NuMA S1" cells with TNF-alpha resulted in a 3-fold increase in apoptosis compared to control cells (S1 cells treated with IgGs following short permeabilization). Western blot analysis revealed that "anti-NuMA S1" cells have upregulated ICE-caspase and that preventing basement degradation by using inhibitors of metalloproteinases does not abolish the upregulation of ICE-caspase and the increase in apoptosis sensitivity (Lelièvre et al., in manuscript form). This suggests that upregulation of caspase pathway precedes or is independent of metalloproteinase activation. Thus NuMA appears to play a key role in maintaining the balance between differentiation and apoptosis.

b) Molecular mechanisms of NuMA function.

The fact that NuMA antibodies could reach the cell nucleus upon simple permeabilization of the membrane of living cells indicates that these antibodies were actively transported to the nucleus. These antibodies were directed against the C-terminus of NuMA and, once in the nucleus, they induced NUMA disorganization. They stayed in the nucleus for at least 5 days. I have used other antibodies raised against the N-terminus of NuMA. These antibodies are also transported to the nucleus; however they do not disrupt NuMA organization and they disappear from the cell nucleus after a couple of days. Time course experiments indicated that these antibodies were transported back to the cytoplasm (figure 2A). The most probable explanation is that the antibodies traveled in and out of the nucleus with NuMA. To test the hypothesis that NuMA protein may be shuttling, I treated S1 cells with actinomycin D, which in a number of cases has been shown to prevent nuclear import. Following a five-hour treatment, NuMA was found in both the cytoplasm and the nucleus, indicating it was indeed traveling between both compartments (figure 2B). I then performed fusion heterokaryon experiments between human S1 cells and mouse NIH-3T3 cells and detected NuMA in both types

of nuclei using an antibody that selectively recognized the human form of NuMA (not shown; unpublished data). This confirms that NuMA is shuttling between the cytoplasm and the cell nucleus. Electron microscopy and soft X-ray microscopy analysis have confirmed the presence of NuMA in the cytoplasm of untreated cells (not shown). The combination of high resolution microscopy and computational search related to NuMA gene and protein sequences have now indicated possible functions for this protein in the regulation of gene expression. Experiments are in progress.

c) <u>NuMA is a marker of cell phenotype</u>.

Since NuMA displays several different distribution patterns depending on the cellular phenotype (e.g., proliferation, growth-arrest, acinar differentiation), we decided to develop assays that would give a quantitative analysis of NuMA organization within the cell nucleus. To do so we started a collaboration with mathematicians (Steve Lockett's group) who are working on imaging analysis at the Lawrence Berkeley National Laboratory. This collaboration has led to the development of a "punctateness" algorithm that can measure differences in the intensity of neighboring pixels and hence significantly identifies alterations in the distribution of a protein based on immunostainings (Figure 3). The algorithm uses nuclear segmentation based on DNA staining to reconstruct the entire nuclear volume. We tested this algorithm on distribution patterns of NuMA that could be discriminated by the eye. When cells go from proliferating to growth-arrested to differentiated (acini), NuMA distribution changes from diffuse to numerous small aggregates to enlarged peripheral foci. This progressive aggregation of NuMA could be easily measured with the punctateness algorithm (Knowles and Lelièvre et al., in manuscript form). Then, we tested the algorithm on NuMA stainings that could not be discriminated by the eye, and that corresponded to different cellular phenotypes. For instance, when non-malignant, malignant and revertant cells are proliferating, NuMA is diffusely distributed in all cases and usual cell cycle markers are upregulated in a similar way, as shown by both immunostaining and western blot analysis. When we applied the algorithm to these images we found significant differences in the degree of "diffusion" of NuMA. NuMA distribution was more diffuse in tumor cells compared to non-malignant cells (figure 4) and revertant RT4 cells showed a degree of diffusion similar to that measured in non-malignant cells (not shown; Knowles and Lelièvre et al., in manuscript form). Moreover, the same analysis performed on tumor cells MDA 231 measured a degree of diffusion similar to that obtained with T4-2 tumor cells. This indicates that quantitative analysis of NuMA staining patterns can discriminate between malignant and non-malignant cells although both cell types are proliferating. In the future, this algorithm will be tested on clinical specimens.

The collaboration with Steve Lockett's group led to another manuscript in which nuclear segmentation is based on peripheral nuclear staining instead of DNA staining (Ortiz de Solorzano et al., submitted). This idea came from results I obtained with lamin staining that clearly showed strong alterations in nuclear shape between non-malignant and malignant cells cultured in 3D rBM (Lelièvre et al., in manuscript form). It is anticipated that nuclear segmentation based on staining for nuclear membrane proteins will be more accurate to measure the nuclear volume.

d) <u>Conclusions</u>:

Completion of this two year project has led to the identification of a number of NMPs that are

potential tumor suppressor or tumor promoter candidates. This part of the project done in collaboration with Dr. Getwenberg was slowed down by the tremendous work that had to be done to cross-analyze many 2D gels prepared from all the steps of the progression series. Therefore the SOW was modified upon request following the first report to illustrate the emphasis on the NuMA project. The study of NuMA in relation with differentiation and tumorigenesis has demonstrated that this protein is an essential regulator of the HMEC phenotype. Its specific role in differentiation has been deciphered and its mechanisms of action are being investigated. The study of NuMA has also led to the development of new imaging techniques to measure alterations of the nuclear organization in tumor cells. It is anticipated that NuMA studies will lead to a better understanding of the regulation of HMEC behavior and to the development of new therapeutic strategies.

The work performed during the funding period has led to the preparation of four research papers (one published, one submitted and two in "manuscript form"). It has also led to the publication of two review papers (one published, one in press) that presented important new concepts about the role played by cell structure, and more particularly nuclear structure, in the regulation of the cellular phenotype.

Based on these results I have now been awarded to grants to work on two different projects focusing on NuMA. The work on NMPs, done in collaboration with Dr. Getzenberg, will be continued in the Bissell laboratory.

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DW Knowles, SA Lelièvre, WS Chou, A Lee, W Wen, C Ortiz de Solorzano, MJ Bissell, and SJ Lockett. Quantitative model-based image analysis of sub-visual changes in NuMA distribution links nuclear organization with cell phenotype. (<u>In manuscript form</u>)

C Ortiz de Solorzano, R. Malladi, SA Lelièvre, and SJ Lockett. Segmentation of nuclei and cells using membrane related protein markers. (<u>Submitted</u>)

Figure 1. NuMA-basement membrane (BM) communication in organized (acini: S1-50, RT4 cells) and disorganized (cell clusters: S1-EGFR, T4-2) structures. Confocal fluorescence images (0.2 μ optical sections) of NuMA (a-d) and collagen IV (e-h) immunostainings. (a-d) NuMA distribution shown in one nucleus (700X magnification): NuMA is distributed in large aggregates at the periphery of the nucleus of S1-50 (a) acinar cells. The protein is diffusely distributed in the nucleus of S1-EGFR cells (b), that form slowly growing cells clusters, and T4-2 cells (c), that form rapidly growing cell clusters. However, when T4-2 cells are induced to form organized acini in 3D rBM culture upon treatment with beta1-blocking or EGFR-blocking antibodies (reversion experiments), NuMA becomes distributed into large aggregates at the nuclear periphery (d). The scheme under the images illustrates NuMA distribution (in gray). (e-h) BM organization in S1-50, S1-EGFR, T4-2, and RT4 cells. Acini formed by S1-50 and RT4 cells, and clusters (no acini formation) formed by S1-EGFR cells are surrounded by an intact collagen IV-rich BM (e, h, and f respectively). The formation of disorganized tumor-like clusters by T4-2 cells is accompanied by an alteration of the deposition of collagen IV (both inside and outside the cell cluster) (g). Some clusters secrete very low amount of collagen IV (not shown). When incubated with anti-NuMA antibody following digitonin permeabilization, 40% of the initial acini population show a degradation of their collagen IV-rich BM (histograms for S1-50 and RT4 cells), whereas no change in the deposition of collagen IV is observed for S1-EGFR and T4-2 clusters (see corresponding histograms). In the case of T4-2 clusters, the evaluation was done by counting the number of spheroids with low or no secretion of collagen IV, compared to spheroids with high secretion of collagen IV. Scale bar is 20 μ.

Figure 2A. Translocation of anti-NuMA antibodies in and out of the nucleus. Confocal fluorescence images (0.2 μ optical sections) of NuMA using an antibody directed against the N-terminus of the protein. Antibodies were introduced into acinar cells (3D-rBM culture) using reversible digitonin permeabilization. Staining done with secondary antibodies indicates that similarly to the antibody directed against the C-terminus of NuMA (Lelievre et al., 1998), the 'N-terminus' antibody translocates into nucleus (a; arrows indicate nuclei). Interestingly it translocates back to the cytoplasm where it accumulates after a short while (b; black holes correspond to the location of nuclei).

Figure 2B. Effect of actinomycin D on NuMA compartmentalization. Confocal fluorescence images (0.2 μ optical sections) of F- actin (a) and NuMA (b,c) in early 3D cultures of HMECs S1-50. F-actin staining indicates the boundaries of cells inside the 3D cluster (a; black holes represent the location of nuclei: N).(b) Control: NuMA is localized in the nuclei. (c) Treatment with actinomycin D (AD) an inhibitor of transcription which has been shown to prevent the nuclear import of proteins: after a five hour treatment with AD (5 μg/ml), NuMA is found in both cytoplasmic and nuclear compartments, as indicated by a diffuse staining in the entire cluster of cells.

Figure 3. Mathematical modeling of the measurement of punctateness.
An algorithm developed by Lockett and colleagues was further adapted to measure the punctateness of staining patterns for the analysis of NuMA organization. A diffuse staining (fine pattern) blurred with defined blur coefficients rapidly loses contrast (a,b,c),

whereas a punctate staining (coarse pattern) blurred with the same coefficient loses contrast more gradually (d,e,f). The degree of contrast plotted in function of the blur factor gives a curve the slope of which is steeper for a more diffuse (or fine) staining. As a result, the curve drawn from a diffuse staining is below the curve drawn from a coarse staining (right panel). It was calculated that the fourth level of blurring on the scale used is sufficient to indicate if there is a significant difference between two staining patterns [Knowles et al., in preparation]. All measurements are automatically normalized for background, staining intensity, and nuclear volume.

Figure 4. Difference in the punctateness of NuMA staining between proliferating non-malignant and malignant cells cultured in 3D.
Proliferating non-malignant S1-50 cells and malignant T4-2 cells (day 3 of 3D culture) were immunostained with anti-NuMA antibody. No significant difference in the staining pattern of NuMA could be detected by direct visualization of an S1-50 or T4-2 nucleus. Topro-3 was used as a counterstaining for DNA, which permitted the nuclear segmentation and the reconstruction of the entire nuclear volume. The measurement of the punctateness of the staining revealed a significant difference in NUMA distribution between non-malignant and malignant cells, as shown by the higher level of contrast for NuMA staining in S1 cells compared to T4-2 cells. The histogram shows results calculated for the fourth blurring step. It is therefore possible to discriminate between non-malignant and malignant cells, even if they are all proliferating by measuring the degree of punctateness of NuMA staining.
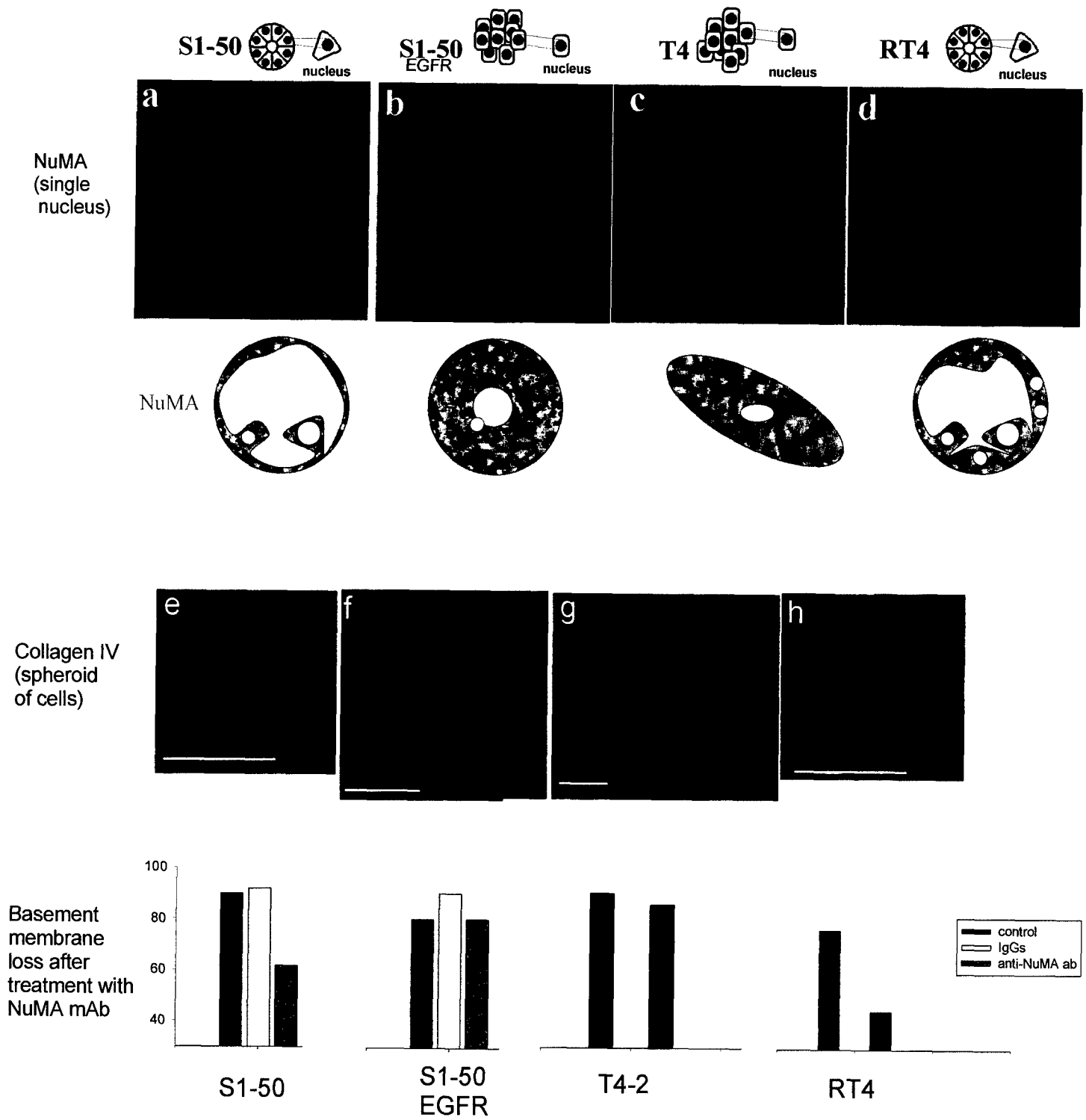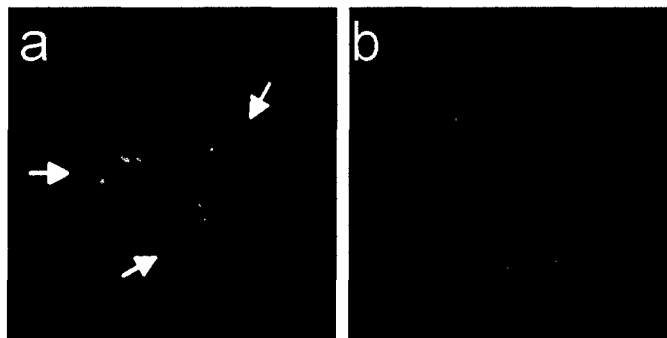
Figure 1

Figure 2

A

NuMA-NT mAB

a	b

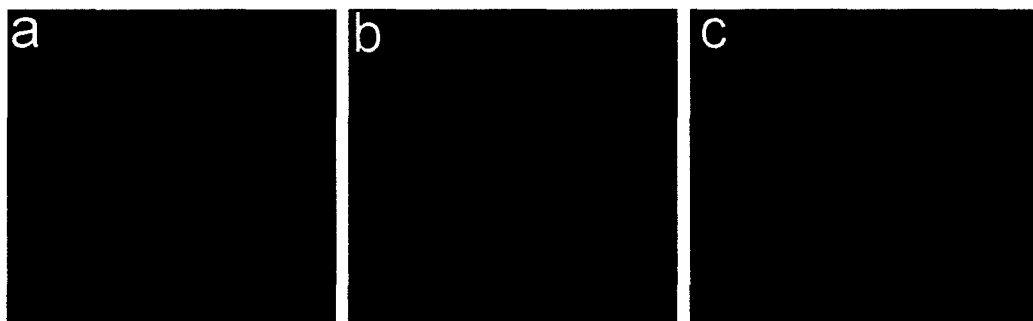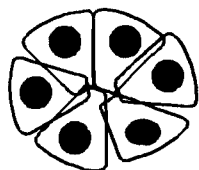12 hours   40 hours

B

F-actin   **NuMA**   **NuMA**

3D culture

control   +Actinomycin D

Figure 3

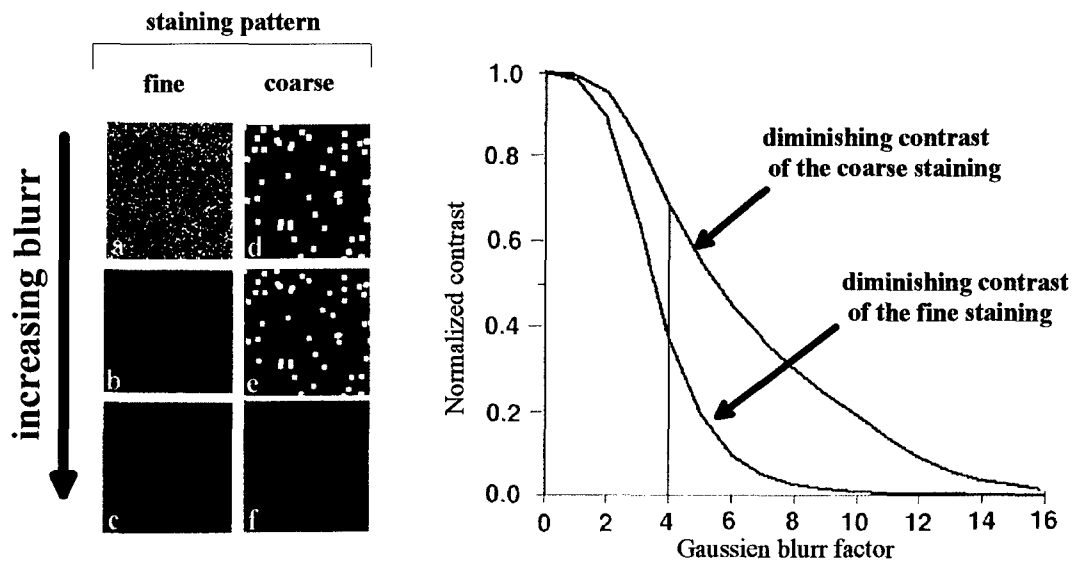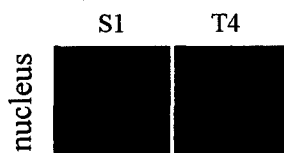## Mathematical modeling of variations in punctate staining patterns using test images
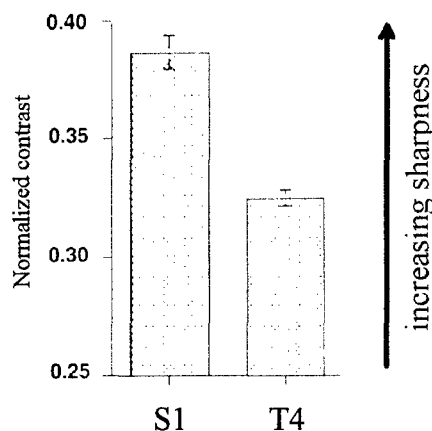

Figure 4

The nuclear distribution of NuMA protein is significantly different between proliferating non-malignant and malignant mammary epithelial cells

APPENDIX 1

BULLETED LIST OF KEY RESEARCH ACCOMPLISHMENT

♦ identification of three potential tumor suppressor candidates based on 2D gel analysis of nuclear matrix associated proteins (NMPs).

♦ identification of three potential tumor promoter candidates based on 2D gel analysis of NMPs.

♦ identification of several NMPs that may play a role in early stages of tumor progression (loss of proper differentiation).

♦ demonstration that nuclear organization is dependent on tissue formation.

♦ demonstration that nuclear organization directs tissue phenotype.

♦ demonstration that NuMA protein is a regulator of mammary epithelial cell differentiation.

♦ demonstration that NuMA protein can translocate to various areas within cells.

♦ establishment of imaging techniques that permit quantitative measurements of the distribution patterns of nuclear proteins.

♦ demonstration that NuMA distribution can be used to discriminate between various cellular phenotypes (e.g., proliferation, growth-arrest, acinar differentiation), and between proliferating non-malignant and malignant cells.

APPENDIX 2

LIST OF REPORTABLE OUTCOMES

Manuscripts related to this project:
1. **Lelièvre SA**, Weaver VM, Nickerson JA, Bhaumik A, Petersen OW, Bissell MJ. Tissue phenotype depends on reciprocal interactions between the extracellular matrix and the structural organization of the nucleus. Proc. Natl. Acad. Sci. (USA) 1998; 95:14711-14716.
2. **Lelièvre SA**, Bissell MJ. Communication between the cell membrane and the nucleus: the role of protein compartmentalization. J. Cell. Biochem. 1998; 30/31:250-64.
3. Bissell MJ, Weaver VM, **Lelièvre SA**, Wang F, Petersen OW, Schmeichel KL. Tissue structure, nuclear organization and gene expression in normal and malignant breast. Cancer Res.(SUPPL) 1999; 59: 1757s-1764s.
4. **Lelièvre SA,** Pujuguet P, Bissell MJ. Cell nucleus in context. Crit. Rev. Eukar. Gene Expression (in press)
5. C Ortiz de Solorzano, R. Malladi, **SA Lelièvre**, and SJ Lockett. "Segmentation of nuclei and cells using membrane related protein markers." (Submitted)
6. Knowles DW, **Lelièvre SA**, Chou WS, Lee A, Wen W, Ortiz de Solorzano C, Bissell MJ, Lockett SJ. Quantitative model-based image analysis of sub-visual changes in NuMA distribution links nuclear organization with cell phenotype. (In manuscript form)
7. **Lelièvre SA**, Weaver VM, Larabell CA, Bissell MJ. The nuclear protein NuMA is a regulator of human breast epithelial cell differentiation. (In manuscript form)

Abstracts related to this project:
1. **S Lelièvre**, WM Weaver, CA Larabell, JA Nickerson, and MJ Bissell. "Nuclear architecture changes as a function of both cell growth and 3-dimensional tissue organization." ASCB meeting, *Proc. Am. Soc. Cell Biol.*, Suppl. Vol.8, p589a, December **1997** (Washington DC)
2. **S Lelièvre**, CA Larabell, W Meyer-Ilse, J Brown, JA Nickerson, A Viron, E Puvion, and MJ Bissell. "Global rearrangement of nuclear matrix-associated proteins when human mammary epithelial cells are cultured in 3-D: an analysis using confocal-, electron-, and soft x-ray microscopy." Keystone Symposium on Nuclear Matrix, p27, April **1998** (Copper Mountain, CO)
3. **SA Lelièvre**, VM Weaver, JA Nickerson, CA Larabell, A Bhaumik, OW Petersen, and MJ Bissell. "Dependence of nuclear structure on tissue organization during extracellular matrix-induced mammary epithelial cell morphogenesis." Cold Spring Harbor Symposium on Nuclear Structure and Function, October **1998** (Cold Spring Harbor)
4. **SA Lelièvre**, VM. Weaver, JA. Nickerson, CA. Larabell, A Bhaumik, OW Petersen, and MJ Bissell."Reciprocal interactions between extracellular matrix, nuclear organization, and tissue phenotype." ASCB meeting, December **1998** (San Francisco, CA)
5. **SA Lelièvre**, VM. Weaver, MJ Bissell. "Nuclear-directed signaling in mammary gland acini", Gordon Conference on Biological Structure and Gene Expression, August **1999** (Meriden, NH)

Dr Sophie Lelièvre's Presentations related to this project:

Speaker at national and international meetings:
"Global rearrangement of nuclear matrix-associated proteins when human mammary epithelial cells are cultured in 3-D: an analysis using confocal-, electron-, and soft x-ray microscopy", Keystone Symposium on Nuclear Matrix (Copper Mountain, CO; junior investigators workshop), April 1998; **"Nuclear structure, cell proliferation, and tissue morphogenesis"**, American Society for Cell Biology Meeting (San Francisco, CA), December 1998; **"Nuclear-directed signaling in mammary gland acini"**, Gordon Conference on Biological Structure and Gene Expression (Meriden, NH; short talk), August 1999.

Invited seminars:
"Dynamic reciprocity between the extracellular matrix and the organization of the cell nucleus: a study of mammary epithelial cell morphogenesis", Institut de Génétique Moléculaire, Paris, France, June 1998; **"Interrelationships between the distribution of nuclear matrix proteins, chromatin structure and gene expression during mammary epithelial cells morphogenesis"**, CEA, Fontenay aux Roses, France, June 1998; **"Communication between the extracellular matrix and the nuclear structure in breast development and malignancy"**, Boston University Medical School, Dept of Biochemistry, February 1999; **"The role of nuclear organization in normal and malignant breast structures"**, California Pacific Medical Center Research Institute (San Francisco, CA), May 1999; **"Nuclear organization in normal and malignant breast"**, Division of Radiation and Cancer Biology, New England Medical Center, TUFTS University, (Boston, MA), August 1999.

Patents related to this project: *"Utilization of nuclear structural proteins for targeted therapy and detection of proliferative and differentiation disorders"*- IB-1454, (applied for)

Development of techniques related to this project:
Use of staining patterns of nuclear proteins to discriminate between different cell phenotypes and between nonmalignant and malignant breast epithelial cells; partly achieved by utilization of a punctateness algorithm (developed in collaboration with David Knowles and Steve Lockett at the Lawrence Berkeley National Laboratory).

Funding applied for:
Boehringer-Ingelheim (awarded 2000-2001)
IDEA grant DOD/BCRP (awarded 2000-2003)

Employment:
Research Scientist (Biologist) in the Life Sciences Division at the Lawrence Berkeley National Laboratory, Berkeley CA, (2000-ongoing)

APPENDIX 3 :

MANUSCRIPTS AND ABSTRACTS

Tissue phenotype depends on reciprocal interactions between the extracellular matrix and the structural organization of the nucleus

Sophie A. Lelièvre[*], Valerie M. Weaver[*], Jeffrey A. Nickerson[†], Carolyn A. Larabell[*], Ankan Bhaumik[*], Ole W. Petersen[‡], and Mina J. Bissell[*§]

[*]Lawrence Berkeley National Laboratory, Berkeley, CA 94720; [†]University of Massachusetts Medical School, Worcester, MA 01655; and [‡]The Panum Institute, DK-2200 Copenhagen N, Denmark

ABSTRACT What determines the nuclear organization within a cell and whether this organization itself can impose cellular function within a tissue remains unknown. To explore the relationship between nuclear organization and tissue architecture and function, we used a model of human mammary epithelial cell acinar morphogenesis. When cultured within a reconstituted basement membrane (rBM), HMT-3522 cells form polarized and growth-arrested tissue-like acini with a central lumen and deposit an endogenous BM. We show that rBM-induced morphogenesis is accompanied by relocalization of the nuclear matrix proteins NuMA, splicing factor SRm160, and cell cycle regulator Rb. These proteins had distinct distribution patterns specific for proliferation, growth arrest, and acini formation, whereas the distribution of the nuclear lamina protein, lamin B, remained unchanged. NuMA relocalized to foci, which coalesced into larger assemblies as morphogenesis progressed. Perturbation of histone acetylation in the acini by trichostatin A treatment altered chromatin structure, disrupted NuMA foci, and induced cell proliferation. Moreover, treatment of transiently permeabilized acini with a NuMA antibody led to the disruption of NuMA foci, alteration of histone acetylation, activation of metalloproteases, and breakdown of the endogenous BM. These results experimentally demonstrate a dynamic interaction between the extracellular matrix, nuclear organization, and tissue phenotype. They further show that rather than passively reflecting changes in gene expression, nuclear organization itself can modulate the cellular and tissue phenotype.

The cell nucleus is organized by a nonchromatin internal structure referred to as the nuclear matrix (NM; refs. 1–3). Identified NM components include coiled-coil proteins (4), cell cycle regulators (5), tissue-specific transcription factors (6, 7), and RNA splicing factors (for review see ref. 2). Although splicing factors have been shown to redistribute during cellular differentiation (8, 9) and following the induction of gene expression (10), spatial distribution of nuclear components are thought to be the consequence of changes in gene expression (8, 10, 11). However, whether NM composition and structure may themselves affect gene expression and cellular function has not been examined.

To systematically study the effect of cell growth and tissue differentiation on nuclear organization, we used a reconstituted basement membrane (rBM)-directed model of mammary gland morphogenesis (12). The HMT-3522 human mammary epithelial cells (HMECs) were isolated from reduction mammoplasty and became immortalized in culture (13). When

embedded within a rBM, these cells arrest growth, organize an endogenous BM, and form polarized acinus-like structures with vectorial secretion of sialomucin into a central lumen (12). We used this model to compare the nuclear organization of HMECs cultured on a plastic surface [two-dimensional (2D) monolayer] vs. a three-dimensional (3D) rBM. Nuclear organization was assessed by examining the distribution of the coiled-coil NM proteins lamin B (14) and NuMA (15), the cell cycle regulator Rb (p110Rb; ref. 5), and the splicing factor SRm160 (formerly known as B1C8; ref. 16). These proteins had distinct spatial distribution patterns specific for proliferation, growth arrest, and acini formation. Moreover, disruption of nuclear organization in acini by either perturbing histone acetylation or directly modifying the distribution of NM proteins altered the acinar phenotype.

We previously hypothesized (17) and thereafter provided evidence that the extracellular matrix (ECM) directs morphogenesis and gene expression in mammary epithelial cells (12, 18, 19). Here we show that a reciprocal relationship exists between the ECM and nuclear organization. These findings underscore a role for nuclear organization in regulation of gene expression and provide a possible framework for how cell–ECM interactions determine cell and tissue phenotype.

MATERIALS AND METHODS

Cell Culture. HMT-3522 HMECs (S-1 passage-50 cells; ref. 13) were propagated in 2D cultures in chemically defined medium (12), and growth arrest was induced by removing epidermal growth factor (EGF) for 48 hr. Cultures were prepared by embedding single cells (8.5×10^5 cells per ml of matrix) in rBM (Matrigel, Collaborative Research) or collagen-I matrix (Cellagen AC-5, ICN) in 4-well chamber slides (Nalge). These cultures were grown for 5–10 days. Growth arrest and morphogenesis were routinely observed by days 7–9.

Antibodies and Inhibitors. For Western blots and/or immunostaining, we used mAbs against type IV collagen (clone CIV, Dako), β-catenin (clone 14, Transduction Laboratories, Lexington, KY), SRm160 splicing factor (clone B1C8, 16), lamin B (clone 101-B7, Matritech, Cambridge, MA), NuMA (clone 204–41, Matritech, and clone B1C11, a gift from S. Penman, Massachusetts Institute of Technology, Cambridge, MA), and polyclonal antibodies (pAbs) against Ki-67 (Novo-Castra, Newcastle, U.K.), acetylated histone H4 (Upstate Biotechnology, Lake Placid, NY), and p110Rb (Santa Cruz Biotechnology). For bioperturbation assays, we used mAbs against lamins A/C (clone 636, Novocastra, Newcastle, U.K.)

Abbreviations: NM, nuclear matrix; BM, basement membrane; rBM, reconstituted BM; HMEC, human mammary epithelial cells; 2D and 3D, two and three dimensional; Rb, retinoblastoma protein; ECM, extracellular matrix; EGF, epidermal growth factor.
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and NuMA (clone 22, Transduction Laboratories, Lexington, KY), in addition to B1C11 and 101-B7. Trichostatin A (Wako Chemicals, Richmond, VA) was used as an inhibitor of histone deacetylase (40 nM).

Indirect Immunofluorescence. Cells were permeabilized *in situ* (0.5% Triton X-100 in 100 mM NaCl/300 mM sucrose/10 mM Pipes, pH 6.8/5 mM MgCl2 containing 1 mM Pefabloc Sc (AEBSF) (Boehringer Manheim)/10 μg/ml leupeptin/10 μg/ml aprotinin/10 μg/ml trypsin inhibitor type II/250 μM NaF), fixed in 2% paraformaldehyde, and immunostained as described (18). Human mammary tissue was snap-frozen in n-hexane and embedded in Tissue-Tek O.C.T. compound (Sakura Firetek, Torrance, CA); 5-μm sections were fixed in methanol and immunostained in accordance with human protocol (KF) 01–216/93 in the laboratory of O.W.P.

Image Acquisition, Processing, and Data Analysis. Samples were analyzed by using a Bio-Rad MRC 1024 laser scanning confocal microscope attached to a Nikon Diaphot 200 microscope. Fluorescence specificity was verified by sequential fluorophore excitation. NuMA foci were analyzed by using IMAGE SPACE-3D analysis program (Molecular Probes) and normalized to 3D rBM cluster-cell number by highlighting and counting each nucleus using IMAGE SPACE-MEASURE 2D. The voxel threshold was set at 0.2 μm.

Immunoblot Analysis. Total cell extracts (2% SDS in phosphate-buffered saline, pH 7.4, containing 1 mM Pefabloc/10 μg/ml leupeptin/10 μg/ml aprotinin/10 μg/ml trypsin inhibitor type II/250 μM NaF) were prepared *in situ* for 2D cultures or from acini isolated from 3D cultures by dispase treatment (5,000 units per ml caseinolytic activity, Collaborative Research). Equal amounts of protein were separated and immunoblotted as described (18).

***In Situ* NM Preparation.** *In situ* NM preparation was as previously described (20), except that 0.05% Triton X-100 and micrococcal nuclease (5 units per ml; Sigma) were used.

Antibody-Mediated Perturbation of Nuclear Organization. rBM-induced acini (day 10) were permeabilized for less than 2 min *in situ* (0.01% digitonin in 25 mM Hepes, pH 7.2/78 mM potassium acetate/3 mM magnesium acetate/1 mM EGTA/300 mM sucrose/1% RIA-grade BSA), rinsed twice in digitonin-free buffer, and incubated in medium containing dialyzed specific or mock mAbs (15 μg/ml) for 48 hr, after which the cells were incubated with fresh medium for an additional 48 hr. Antibody concentrations and incubation times were determined empirically. Trypan blue dye-exclusion tests and apoptosis studies verified the absence of digitonin toxicity.

RESULTS

Internal Nuclear Organization Is Remodeled When HMECs Are Cultured Within a Basement Membrane. HMT-3522 HMECs, like primary HMECs, undergo morphogenesis to form tissue-like acini when cultured in a 3D rBM (12, 18). Neither cell type undergoes acinar differentiation when cultured as 2D monolayers. In proliferating 2D cultures, NuMA was diffusely distributed in the nucleus (Fig. 1*b*) except when localized to the spindle poles in mitotic cells (15), and splicing factor SRm160 was distributed into numerous speckles of heterogenous sizes (Fig. 1*c*; ref. 16). In rBM-induced acini, NuMA was redistributed into an average of eight nuclear foci (ranging from 1 to 1.6 μm in diameter) surrounded by diffusely localized NuMA protein (Fig. 1*e*), and SRm160 was distributed into an average of seven large speckles (Fig. 1*f*). In contrast, lamin B maintained a peripheral ring-like distribution around the nucleus, with some internal localization, regardless of culture conditions (Fig. 1 *a* and *d*). The distribution pattern of these proteins was conserved in NM preparations *in situ*, where chromatin was removed before immunolocalization (staining is shown for 3D rBM cultures only (Fig. 1 *g–i*).
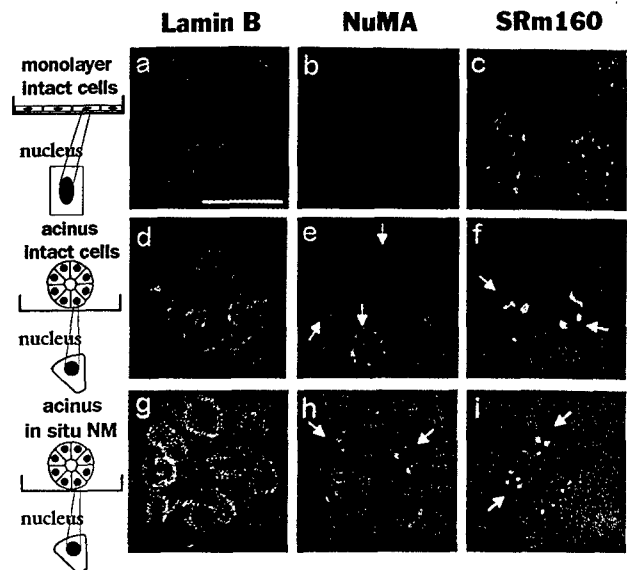

FIG. 1.   NM protein redistribution in HMECs after 3D rBM-induced acinar morphogenesis. Confocal fluorescence images (0.2-μm optical sections) of lamin B, NuMA, and splicing factor SRm160 in cells grown as 2D monolayers (*a–c*) and within 3D rBMs (*d–i*). NuMA was diffusely distributed in the nuclei of cells grown as monolayers (*b*), but reorganized into large nuclear foci in cells induced to undergo morphogenesis (acini formation) in response to a rBM (*e*). SRm160 was distributed as multiple nuclear speckles in cells cultured as monolayer (*c*), whereas it was concentrated into fewer and larger speckles in the acini (*f*). Lamin B, in contrast, consistently localized to the nuclear periphery and within intranuclear patches (*a* and *d*). The distribution of lamin B (*g*), NuMA (*h*), and SRm160 (*i*) after *in situ* NM preparation of cells cultured in 3D rBM was similar to that observed in intact cells (*d–f*). Arrows indicate nuclei found within the plane of the section. (Bar = 10 μm.)

We next examined NuMA and SRm160 distribution at different stages of 3D rBM-induced morphogenesis. After embedment in rBM, cells proliferated to form small clusters by days 3–5 but lacked β-catenin at cell–cell junctions, and collagen IV staining was discontinuous (Fig. 2 *Aa–Ac*). After growth arrest (days 6–10), cells assembled a continuous endogenous BM and formed polarized acinus-like structures with organized adherens junctions (Fig. 2 *Ad–Af*). NuMA was uniformly distributed in the nuclei of proliferating cells (Fig. 2*Ba*), but became concentrated into distinct foci of differing sizes after growth arrest (day 7; Fig. 2*Bb*), and into larger and fewer foci on completion of morphogenesis (day 10, Fig. 2*Bc*). NuMA and the splicing factor SRm160 were not colocalized in proliferating cells (Fig. 2 *Ba'* and *Ba''*), but NuMA foci and SRm160 speckles were closer together after growth arrest (Fig. 2 *Bb'* and *Bb''*) and were completely colocalized in large assemblies after the completion of morphogenesis (Fig. 2*B*, c' and c''). These spatial changes in NuMA arrangement occurred without significant modifications in the level of NuMA expression or molecular weight, as determined by using Western blot analysis (Fig. 2*Be*). These experiments demonstrate that specific NM proteins undergo spatial rearrangement during rBM-induced acinar morphogenesis. Because the existence of NuMA in differentiated tissue has been questioned (21), we studied NuMA in the normal resting human mammary gland. Intense staining was observed in the epithelial cells of acini and ducts, where NuMA was distributed in foci of different sizes and resembled the acinar stages recapitulated in 3D rBM cultures (Fig. 2*Bd*).

Growth Arrest Is Associated With Changes in NuMA and Rb Distribution. ECM-directed growth arrest is an early and critical step in mammary epithelial cell morphogenesis (12). To distinguish between the effect of ECM-directed growth arrest
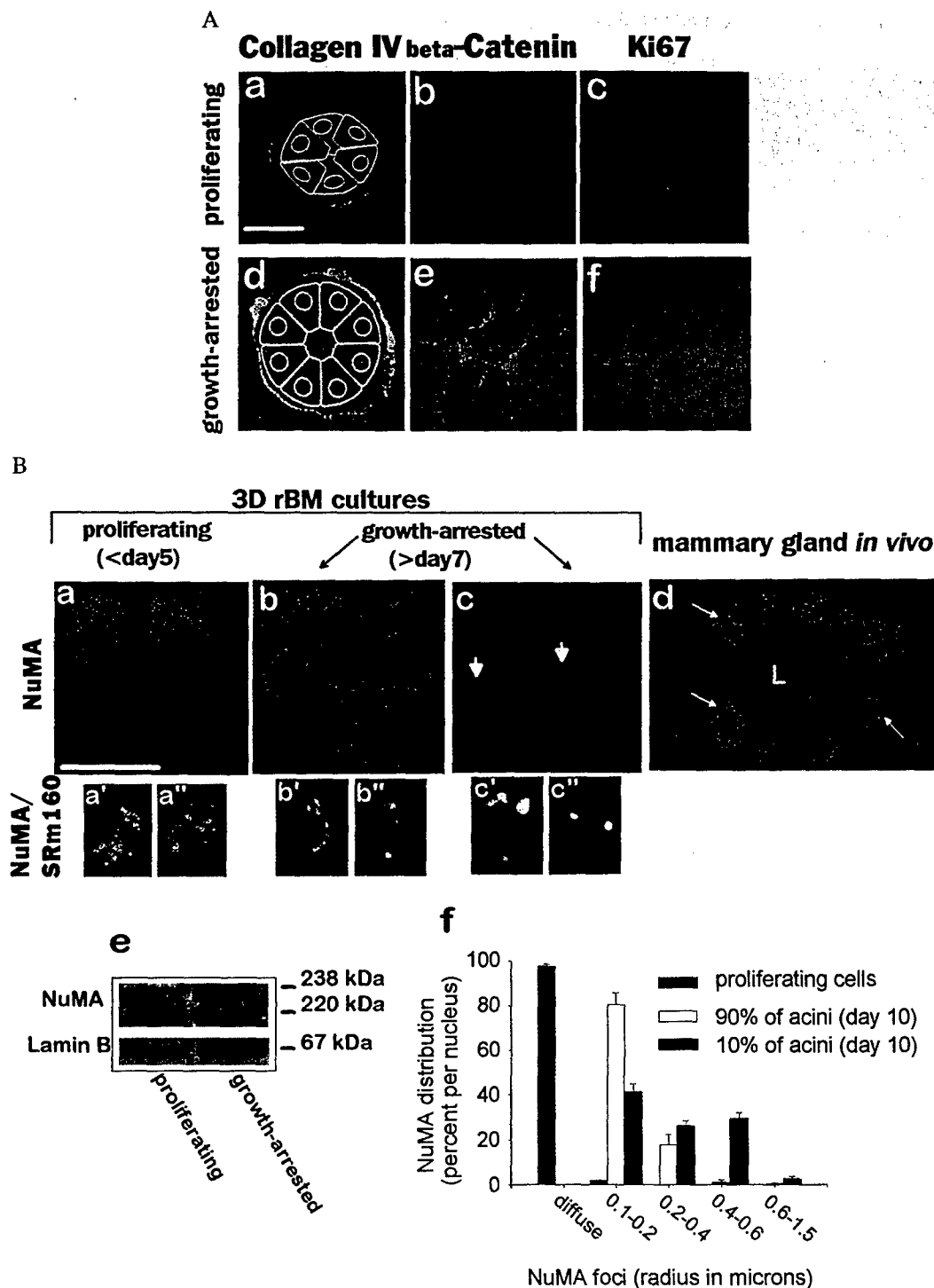
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Proc. Natl. Acad. Sci. USA 95 (1998)   14713

FIG. 2. (*A*) Distribution of structural proteins during rBM-induced acinar morphogenesis. Confocal fluorescence images (0.2-μm optical sections) of collagen IV, β-catenin, and Ki-67 in HMECs embedded within a rBM for 3–4 days (proliferating cells; *a–c*), and for 7–10 days (growth-arrested acini; *d–f*). Coincident with growth arrest and acinar morphogenesis, HMECs deposited an organized endogenous collagen IV-rich BM (*a* vs. *d*), whereas β-catenin relocalized from the cytosol and basal plasma membrane to sites of cell–cell adhesion (*b* vs. *e*). Acinar morphogenesis was associated with cell cycle exit, as indicated by the loss of Ki-67 staining (*c* vs. *f*). (*B*). Spatial analysis of NuMA and splicing factor SRm160 redistribution during rBM-induced acinar morphogenesis. Confocal Texas red fluorescence images (0.2-μm optical sections) of NuMA (*a–c*) and double-labeled NuMA (Texas red), and fluorescein isothiocyanate (FITC) green-stained SRm160 (*a'*, *a''*, *b'*, *b''*, *c'*, and *c''*) in HMT-3522 cells proliferating (a, a', and a'') and undergoing morphogenesis (*b*, *b'*, *b''*, *c*, *c'*, and *c''*) in response to a rBM. In proliferating cells, NuMA was diffusely distributed (*a*) and did not colocalize with SRm160 (*a'* and *a''*). After growth arrest, NuMA coalesced into foci of increasing size (0.2–2 μm; *f*) in association with the establishment of mature tissue-like structures (acini; *b* and *c*) Nine nuclei are shown in *b*. Only the larger NuMA foci observed in late morphogenesis fully colocalized with SRm160 (*b'*, *b''*, *c'*, and *c''*). (*d*) In the ductal and acinar HMECs of the mammary gland, *in vivo*, NuMA was localized in foci with a size distribution comparable to that observed in most of the HMEC nuclei of differentiating rBM cultures shown in *b*. (*e*) Western blot analysis of NuMA and Lamin B showed no difference in protein expression or size between proliferating and growth-arrested HMECs grown within rBMs. Arrows indicate nuclei. (Bars = 10 μm.)

and changes caused by tissue structure and polarity, the localization of NuMA and SRm160 was compared between growth-arrested and proliferating cells cultured in monolayers. Less than 5% of the cells remained in the cell cycle after
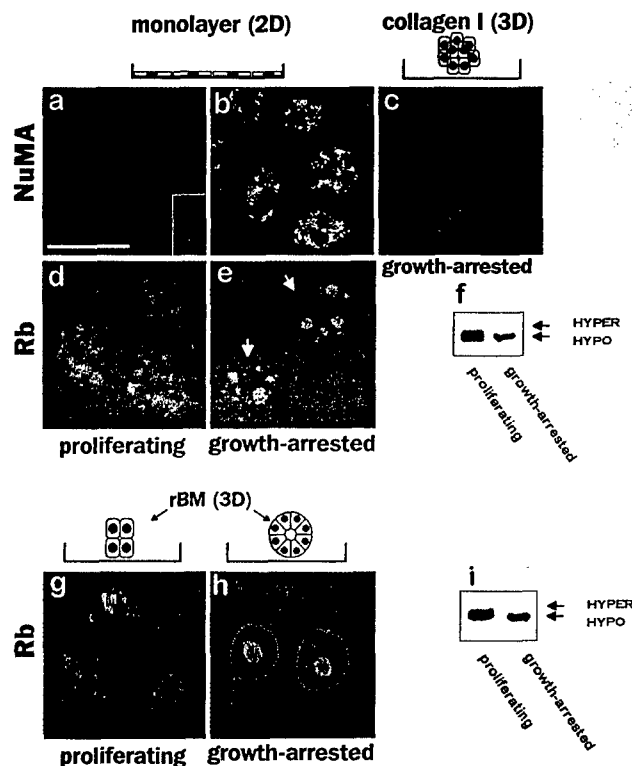
FIG. 3. Effect of growth status on the distribution of NM proteins. Confocal fluorescence images (0.2-µm optical sections) of Texas red-stained NuMA (*a–c*) and fluorescein isothiocyanate (FITC) green-stained Rb (*d, e, g,* and *h*) in cells proliferating as 2D monolayers (*a* and *d*) and within 3D rBMs (*g*) and cells growth-arrested in monolayer (*b* and *e*) and within collagen-I (*c*) or a rBM (*h*). NuMA was diffusely distributed in the nucleus of proliferating HMECs grown as monolayers (*a*) and reorganized into random aggregates on growth arrest induced by EGF removal (*b*). The settings for image recording were the same as for *a*. Aggregates appear white because of saturation of the signal. NuMA was distributed in random aggregates or in small foci in growth-arrested and BM-free cell colonies obtained after 10 days of culture within collagen-I (*c*). Rb was diffusely distributed in the nucleus of proliferating cells grown either in monolayer (*d*) or in 3D rBM (*g*); however, on growth arrest, the protein redistributed into several foci in the monolayer propagated cells (*e*) but coalesced into a central, single nuclear focus in the rBM-induced acini (*h*); the dotted line indicates outer nuclear limit. Western blot analysis of Rb in proliferating and growth-arrested cells grown as monolayers (*f*) or within a 3D rBM (*i*) shows that the hyperphosphorylated isoform was present only in proliferating cells. Arrows indicate nuclei. (Bar = 10 µm.)

growth arrest induced by EGF removal, as indicated by the absence of detectable Ki-67 immunostaining (data not shown). NuMA was uniformly distributed in the nuclei of proliferating cells but coalesced into denser areas on growth arrest (Fig. 3 *a* and *b*). The irregular geometric quality of these dense areas was distinct from the circular foci pattern observed in growth-arrested 3D rBM-grown cells. In contrast, no significant change in the multispeckled distribution of SRm160 was detected under these conditions (data not shown). The relationship between nuclear organization and growth status was further investigated by examining the distribution of the cell cycle regulator Rb. Rb redistributed from a diffuse nuclear pattern in proliferating HMECs into a few large foci in growth-arrested cells (Fig. 3 *d* and *e*). Strikingly, the distribution of Rb in the growth-arrested 2D cultures was distinct from that observed in the growth-arrested 3D cultures (compare Fig. 3 *e* and *h*), which may reflect differences in the state of growth arrest between 2D monolayer and 3D rBM cultures. The monofocal pattern of Rb observed in 3D culture coincided with growth arrest. Western blot analysis showed that hypo-

phosphorylated Rb was associated with the NM in 3D cultures (data not shown) as was previously reported for 2D cultures (5). Moreover, the diffuse distribution observed in proliferating cells was associated with the hyperphosphorylated form of the protein (Fig. 3 *f* and *i*).

Because growth arrest in 3D rBM precedes the final stages of acinar morphogenesis (12), we examined the relationship between the large NuMA foci and the formation of a polarized endogenous BM. HMECs cultured in a 3D collagen-I matrix form growth-arrested organized colonies but do not assemble a polarized, endogenous BM (22). Therefore, we compared NuMA distribution in cells grown in rBM to those grown in type I collagen. After 12 days in collagen I, NuMA was distributed as small foci or irregular dense aggregates (Fig. 3*c*), similar to the pattern observed in growth-arrested cells in 2D cultures. Thus, NuMA redistribution into dense areas and small foci is induced by growth arrest, but the coalescence of the foci into larger and distinct structures requires the presence of a BM.

Cross-Modulation Between NuMA Distribution, Chromatin Structure, and the Acinar Phenotype. The degree of histone acetylation has been shown to regulate chromatin structure and gene expression (19, 23). Histone acetylation was altered in the acini by using the histone deacetylase inhibitor trichostatin A. After 2 hr of treatment, NuMA foci began to disperse, and several cells entered the cell cycle, as measured by an increase in the Ki-67 labeling index. After 24 hr of treatment, NuMA was diffusely distributed in all nuclei (Fig. 4 *e* vs. *a*), and the acinar phenotype was altered as shown by loss of the endogenous BM (Fig. 4 *f* vs. *b*), redistribution of β-catenin (Fig. 4 *g* vs. *c*), and the presence of mitotic cells, as shown by mitotic spindle-pole staining of NuMA (Fig. 4*e*, arrow). In contrast, trichostatin A did not alter the cell phenotype or the distribution of NuMA (data not shown).

Because NuMA is essential for postmitotic nuclear assembly and participates in the loss of nuclear integrity during apoptosis (24, 25), we asked whether disruption of NuMA foci in the acini could globally influence nuclear organization and affect the acinar phenotype. Rapid and reversible digitonin permeabilization was used to load cells with either anti-NuMA mAbs or with an IgG₁ mock mAb. The NuMA mAb B1C11, but not an N-terminal-specific mAb (clone 22; data not shown), disrupted NuMA organization, causing the protein to become diffusely redistributed within the nucleus as revealed by the secondary Ab (Fig. 4*i*). Chromatin structure was altered, as shown by the rearrangement of acetylated histone H4 distribution (Fig. 4 *l* vs. *d*). More dramatically, disruption of NuMA organization altered the acinar phenotype, as indicated by loss of the endogenously deposited BM (Fig. 4*j*). Because the loss could be prevented by treatment with GM6001, a potent metalloprotease inhibitor (Fig. 4*n*; ref. 26), we conclude that NuMA disruption led to induction and/or activation of a metalloprotease. Similar treatment of the acini with mAbs against lamins A/C or lamin B did not induce any change in histone H4 acetylation, BM integrity, or lamin distribution, even though these Abs reached their nuclear targets, as shown by secondary Ab staining (Fig. 4*n* and data not shown).

DISCUSSION

By modifying the cellular microenvironment, we have demonstrated that nuclear organization rearranges dramatically in HMECs after growth arrest and tissue-like acinar morphogenesis (Scheme 1). The use of the 3D-rBM culture assay has enabled us also to show that alterations of nuclear organization can modify the cellular and tissue phenotype.

Previously documented changes in nuclear organization have been broadly descriptive. By systematically analyzing the distribution of three NM proteins in 2D and 3D cultures, we have determined that precise nuclear rearrangements occur
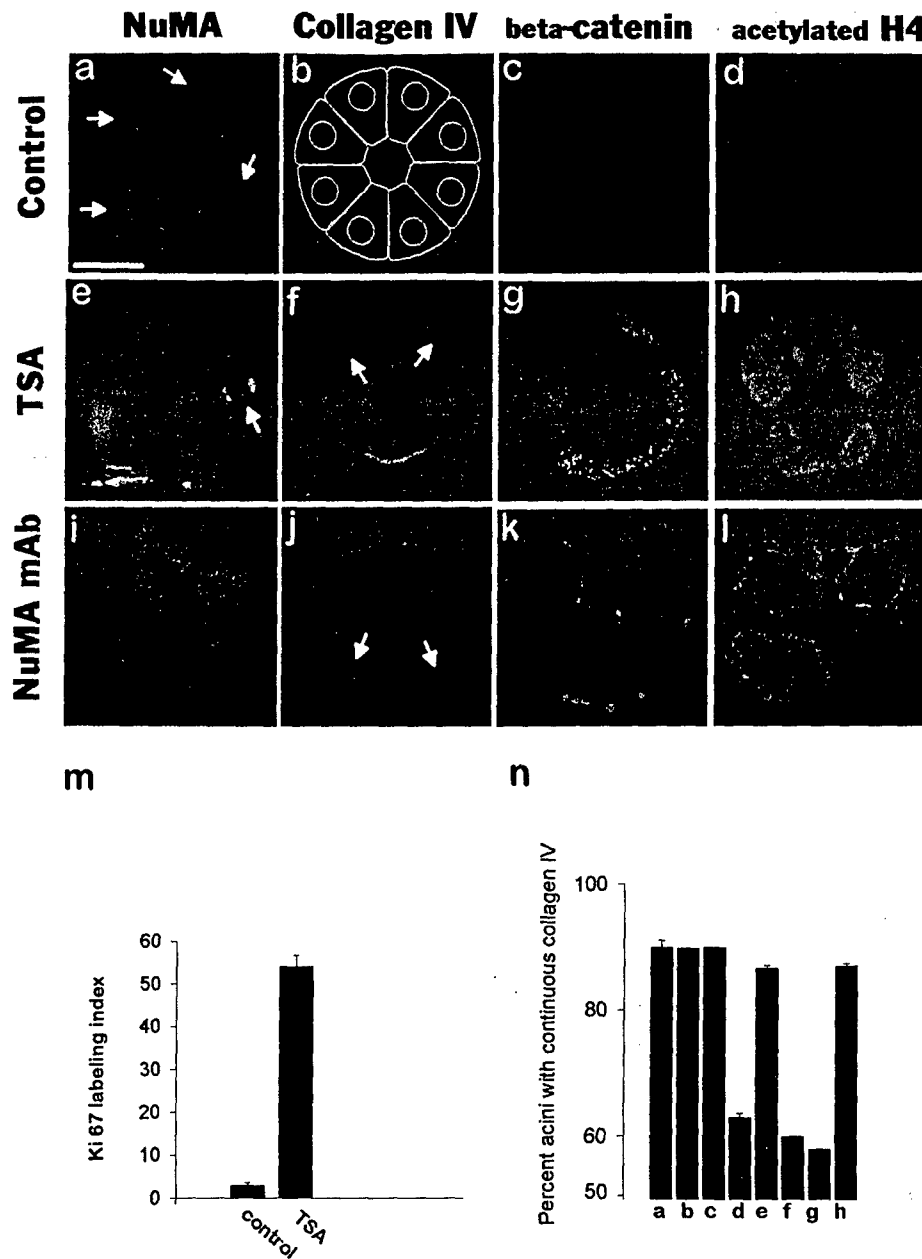
FIG. 4. Cross-modulation between chromatin structure, NM organization, and the acinar phenotype. Confocal fluorescence images (0.2-μm optical sections) of NuMA (a, e, and i), collagen IV (b, f, and j), β-catenin (c, g, and k), and acetylated histone H4 (d, h, and l) in control, trichostatin A (TSA)-treated, and NuMA mAb-incubated acini (day 10 of 3D rBM culture). (a–d) Nuclear organization and acinar phenotype in controls. Acini exhibit NuMA foci (a), an organized endogenous collagen IV-rich BM (b), cell–cell localized β-catenin (c), and dispersed acetylated H4 histone (d). (e–h) Effects of TSA on nuclear architecture and acinar phenotype. After 24 hr of TSA treatment (40 nM), >55% of the cells entered the cell cycle, as indicated by an increase in Ki-67 labeling index (m) and the appearance of mitotic cells (arrow in e). NuMA was uniformly distributed in the nuclei (e), collagen IV disappeared (f), β-catenin was released from the cell–cell interface (g), and the pattern of histone H4 acetylation was altered (h). (i–l) Effects of mAb-induced NuMA foci disruption on nuclear organization and acinar phenotype. Introduction of a NuMA mAb into the nuclei of the acini by using reversible digitonin permeabilization led to the disruption of NuMA foci (i), degradation of the collagen IV-rich BM (arrows in j), and the nuclear marginalization of acetylated H4 histone (l). There was no consistent alteration observed for β-catenin other than increased basal labeling (k). These effects were not observed with mock IgGs or mAbs to lamins A/C or B. (n) BM degradation after mAb-induced NuMA disruption in acini. Analysis of the percentage of acini with intact collagen IV-rich BMs in relation to control/digitonin-permeabilized (DP) acini (a), mock-IgG mAb-treated/DP acini (b), NuMA mAb-treated/nonpermeabilized acini (c), NuMA mAb-treated/DP acini (d), NuMA mAb-treated/DP acini + the metalloproteinase inhibitor GM6001 (e), NuMA mAb-treated/DP acini + the inactive metalloproteinase inhibitor GM1210 (f), NuMA mAb-treated/DP acini + the uPA inhibitor, aprotinin (g), and Lamin B mAb-treated/DP acini (h). Acini (>35%) degraded their endogenous BMs in response to disruption of NuMA (d). The BM loss could be rescued by treatment with the metalloproteinase inhibitor GM6001 (e), but not its inactive analogue (f) or a uPA protease inhibitor (g). (Bar = 10 μm.)

with growth arrest and after rBM-induced morphogenesis. In 3D rBM cultures, both NuMA and Rb were diffusely distributed in the nucleus of proliferating cells. After growth arrest, NuMA was relocalized into discrete foci, whereas Rb redistributed into a central nuclear mass. These patterns of distribution were different from those observed in growth-arrested cells in monolayer 2D cultures, suggesting that there may be different states of growth arrest in 2D and 3D rBM cultures (27). Because NuMA distribution in 3D collagen I cultures was comparable to that observed in growth-arrested 2D cultures, our results suggest that 3D organization of cells per se cannot explain the differences seen between monolayer and 3D rBM

Dynamics of the distribution of NM proteins in 3D rBM

(Only the cell nucleus is represented, NuMA in red; Rb and SRm160 in green)
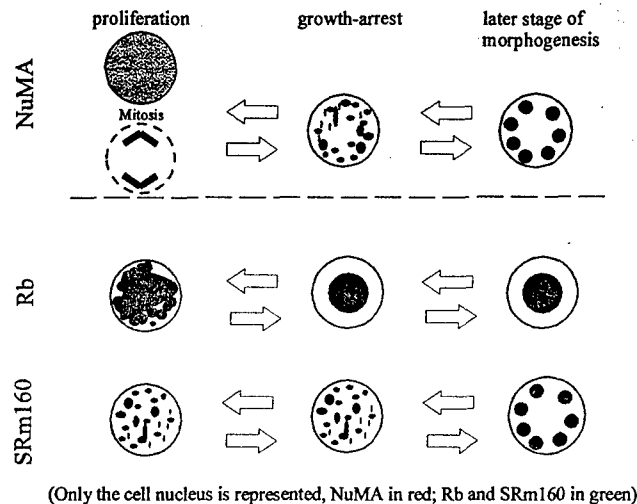
SCHEME 1

cultures. This finding implies that BM signaling is necessary for the ultimate nuclear organization within the acini. Indeed, the presence of large and distinct NuMA foci was observed only in mature 3D rBM cultures and in adult resting mammary gland *in vivo,* where the acini were surrounded by a continuous endogenous BM. The mammary gland undergoes developmental cycles of growth and differentiation even in adults; this may account for the heterogeneity of foci size observed *in vivo* and may further explain the absence of the very large NuMA foci in subpopulations of differentiated acini (Fig. 2*Bc*). Whether the pattern of NuMA distribution indeed corresponds to different levels of differentiation *in vivo* requires further analysis.

The antibody-directed disruption of NuMA foci in the acini induced changes in the distribution pattern of acetylated histone H4, the activation of metalloprotease(s), and the loss of BM integrity. These results, as well as our observation that NuMA progressively coalesces and eventually colocalizes with enlarged splicing-factor speckles during acini differentiation, suggests that some nuclear proteins may contain the molecular information necessary for the development and/or maintenance of the acinar phenotype. Interestingly, trichostatin-induced alteration of histone acetylation in acini also led to the disruption of NuMA foci and was associated with the loss of BM and the induction of cell proliferation. Although we do not know the molecular mechanisms responsible for phenotypic alterations induced by nuclear reorganization, our experiments demonstrate also the existence of reciprocal interactions between nuclear organization, chromatin structure, and the acinar phenotype. The BM has been shown previously to be necessary for the formation and maintenance of the functional acinus (12, 28, 29). We report here that BM-induced acinar formation is associated with the distinct spatial organization of a repertoire of NM proteins and that, conversely, perturbation of nuclear organization alters the BM and influences the acinar phenotype. These results illustrate the dynamic reciprocity between the ECM and the structural organization of the nucleus, and underscore the importance of ECM–NM communication (17) in phenotypic plasticity.

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Communication Between the Cell Membrane and the Nucleus: Role of Protein Compartmentalization

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Abstract   Understanding how the information is conveyed from outside to inside the cell is a critical challenge for all biologists involved in signal transduction. The flow of information initiated by cell-cell and cell-extracellular matrix contacts is mediated by the formation of adhesion complexes involving multiple proteins. Inside adhesion complexes, connective membrane skeleton (CMS) proteins are signal transducers that bind to adhesion molecules, organize the cytoskeleton, and initiate biochemical cascades. Adhesion complex-mediated signal transduction ultimately directs the formation of supramolecular structures in the cell nucleus, as illustrated by the establishment of multi complexes of DNA-bound transcription factors, and the redistribution of nuclear structural proteins to form nuclear subdomains. Recently, several CMS proteins have been observed to travel to the cell nucleus, suggesting a distinctive role for these proteins in signal transduction. This review focuses on the nuclear translocation of structural signal transducers of the membrane skeleton and also extends our analysis to possible translocation of resident nuclear proteins to the membrane skeleton. This leads us to envision the communication between spatially distant cellular compartments (i.e., membrane skeleton and cell nucleus) as a bidirectional flow of information (a dynamic reciprocity) based on subtle multilevel structural and biochemical equilibria. At one level, it is mediated by the interaction between structural signal transducers and their binding partners, at another level it may be mediated by the balance and integration of signal transducers in different cellular compartments. J. Cell. Biochem. Suppls. 30/31:250–263, 1998.   © 1998 Wiley-Liss, Inc.

Key words: signal transduction; cell adhesion complexes; membrane skeleton; nucleo-cytoplasmic translocation

The communication between the cellular microenvironment and the cell nucleus is critical to understanding the essence of cell behavior and tissue development. Signals to be transduced to the cell's interior upon attachment to extracellular matrix (ECM) components, or contact with another cell, are mediated by a variety of adhesion molecules. Using cells cultured in the presence of a reconstituted basement membrane, investigators at a number of labora-

tories have been able to show that the interaction between adhesion molecules of epithelial cells and basement membrane components induces a differentiation program that leads to the formation of functional tissue-like structures [Bissell et al., 1987; Li et al., 1987; Barcellos-Hoff et al., 1989; Caron, 1990; Petersen et al., 1992; Matter and Laurie, 1994; Hoffman et al., 1995]. Similarly, the interaction between adhesion molecules of osteoblasts and fibronectin promotes the production of osteopontin, one of the predominant proteins of the bone tissue [Carvalho et al.,1998]. ECM-mediated regulation of the expression of a defined repertoire of genes is conveyed, at the molecular level, by a modification of DNA-protein interactions [Owen et al., 1990] and the activation of ECM-response elements located in the promoters of some of the expressed genes [Schmidhauser et al., 1990, 1992; Liu et al., 1991]. Cell-cell interaction also induces the expression of specific genes that regulate tissue differentiation and morphogenesis [Takeichi, 1995; Gumbiner, 1996; Redfield et al., 1997]. Moreover, the interplay between the formation and loss of adhe-

sion complexes and the correct balance of different kinds of adhesion molecules is essential for tissue development and maintenance of differentiation [Martins-Green and Bissell, 1995; Herminston et al., 1996], and the alteration of this equilibrium can lead to extreme behavior such as apoptosis [Tenniswood et al., 1992; Sympson et al., 1994; Frisch and Francis, 1994; Boudreau et al., 1995] and tumor formation [Gamallo et al., 1993; Sympson et al., 1995; Lochter and Bissell, 1995; Perl et al., 1998; Hagios et al., 1998].

Whereas it is now well established that cell-ECM and cell-cell interactions can regulate gene expression and cell behavior, the way information is transduced from the cell membrane to the nucleus remains an exciting challenge for biologists to solve at the dawn of the third millennium. Explored mechanisms of cell adhesion-mediated signal transduction encompass the induction of biochemical cascades that ultimately regulate the activity of transcription factors [Baichwal et al., 1991; Juliano and Haskill, 1993; O'Neill et al., 1994], alterations of histone acetylation [Loidl, 1994; Alberts et al., 1998; Myers et al., 1998], and remodeling of supramolecular organization of nuclear matrix proteins, defined by the redistribution of these components to distinct nuclear sites [Lelièvre et al., 1998]. In addition, the existence of a structural continuity from the cell membrane and cytoplasm to the nuclear matrix and chromatin [Capco et al., 1984] has been postulated to also participate in the rapid transmission of information within the cell [Bissell et al.,1982; Ingber, 1997]. Elements of proof for this concept have been brought to our attention during the past decade [Pienta and Coffey, 1992; Sims et al, 1992; Maniotis et al., 1997; Lelièvre et al., 1998].

Cell adhesion-mediated signal transduction is initiated by the supramolecular organization of adhesion molecules and proteins localized in the inner part of the cell membrane, referred to as the membrane skeleton [Luna and Hitt, 1992]. By acting both as inducers of biochemical cascades and as organizers of cytoskeletal fibers, proteins of the membrane skeleton bring together the chemical and mechanical aspects of intracellular signaling. Recently, their role in signal transduction has been underscored by their capacity to travel to the nucleus. The nuclear translocation of membrane skeleton proteins and, inversely, the possibility that proteins usually involved in supramolecular structures of the nucleus may translocate to the cell membrane, raise new and important mechanistic issues for signal transduction, and add a new dimension to the concept of dynamic reciprocity proposed almost two decades ago [Bissell et al., 1982].

STRUCTURAL SIGNAL TRANSDUCERS OF THE MEMBRANE SKELETON

Communication between spatially separated elements such as the cell membrane and the chromatin requires intracellular mediators, referred to as structural and biochemical signal transducers. Our general understanding is that a signal transducer can receive a signal and transfer the information to the next component of the signaling cascade by altering its molecular state and modifying its binding to other cellular components. Typically, signals initiated at cell adhesion sites, by cell-ECM or cell-cell contacts, are transduced by membrane skeleton proteins, also referred to as junctional plaque proteins [Kartenbeck et al., 1982; Ben-Ze'ev, 1997]. Although these structural signal transducers share common characteristics such as involvement in phosphorylation/dephosphorylation cascades and the capacity to induce cytoskeletal reorganization [for review, see Longhurst and Jennings, 1998], they are specific for each type of adhesion complex. Focal adhesions (FAs), a class of cell-ECM adhesion complexes formed by the interaction of various types of integrin heterodimers with specific ECM molecules, contain a large number of connective membrane skeleton (CMS) proteins (e.g., α-actinin, talin, tensin, vinculin, Cas, moesin, fimbrin, paxillin, and zyxin) [Weisberg et al., 1997; Brugge, 1998] that interact with a broad range of kinases and phosphatases, and are implicated in the control of actin and myosin filament assembly (Fig.1) [Schlaepfer and Hunter, 1996; Golsteyn et al., 1997; Helmke et al., 1998]. Similarly, hemidesmosomes are formed by the interaction between the ECM component laminin and $\alpha 6$-$\beta 4$ integrin, and the recruitment of CMS proteins, plectin and bullous pemphigoid antigen 230, to form the hemidesmosomal plaque. Hemidesmosome formation directs the organization of intermediate filament type proteins and initiates phosphorylation cascades [Wiche et al., 1993; Giancotti, 1996; Jones et al., 1998; Rezniczek et al., 1998; Schaapveld et al., 1998] (Fig. 1). Cell-cell adhesion complexes (i.e.,
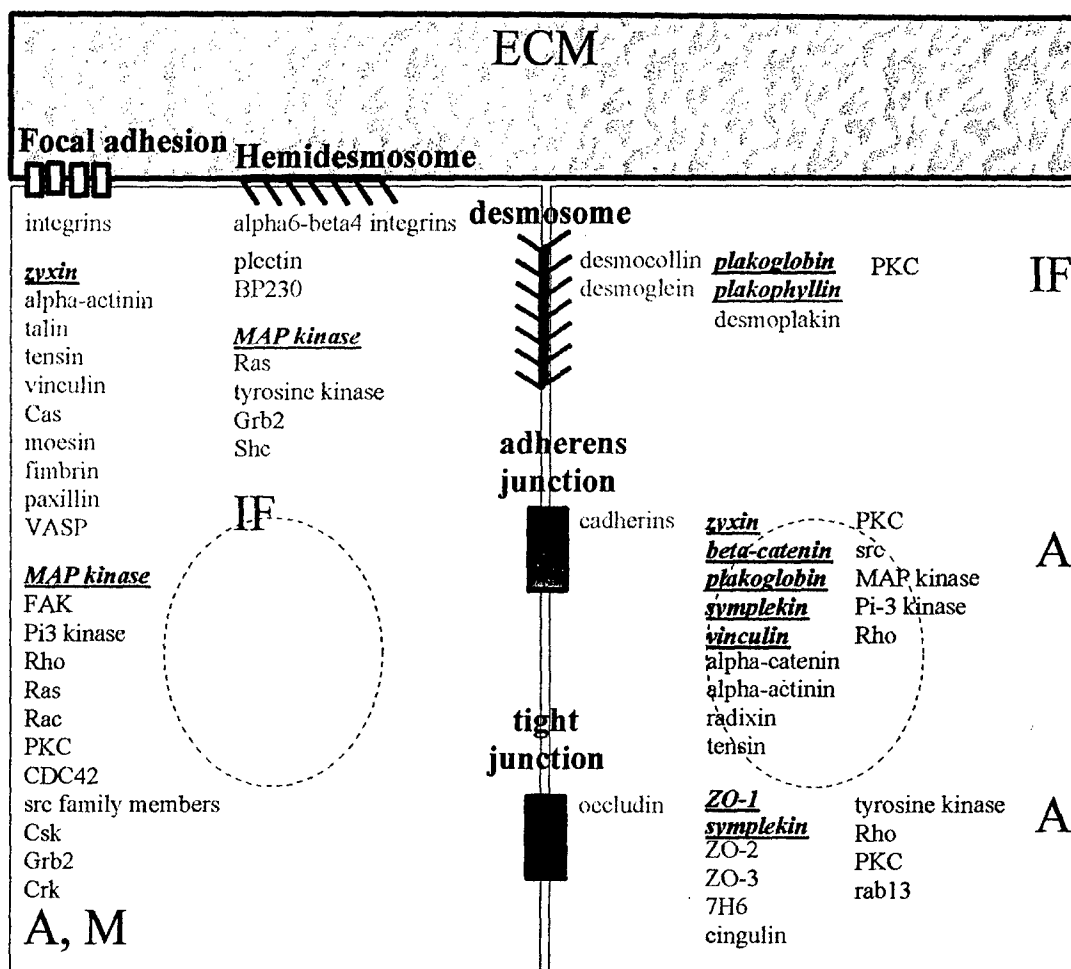
Fig. 1. Organization of cell-cell adhesion complexes (tight junction, adherens junction, desmosome) and cell-ECM adhesion complexes (focal adhesion, hemidesmosome). The formation of cell-cell and cell-ECM interactions involves specific cell membrane adhesion molecules (*blue*), that interact with connective membrane skeleton (CMS) proteins (*green*). CMS proteins themselves interact with other cytoskeletal proteins to organize cytoskeleton networks (A: actin microfilaments; IF: intermediate filaments; M: myosin filaments). Moreover, biochemical signal transducers (*purple*) are found at the cell membrane or in its vicinity and participate in the regulation of cell adhesion complexes and/or in the transfer of signals initiated at cell adhesion complexes. Some CMS proteins (*red*), as well as biochemical signal transducers (*red*), have been observed in the nucleus under specific circumstances. The list of cell adhesion complex components is nonexhaustive. **Color plate on page 329.**

tight junctions, adherens junctions and desmosomes) have been characterized both microscopically [Farquhar et al., 1963] and by their composition. Their localization in a tissue is highly ordered. Tight junctions or zonula occludens are located at the outermost edge of the intercellular space (i.e., at the apical part of epithelial glandular cell assemblies) and are believed to participate in maintaining cell polarity [Cereijido et al., 1998] along with other cell-cell adhesion complexes [Nathke et al., 1994]. Several tight junction-associated CMS proteins have been identified, including ZO-1, ZO-2 and ZO-3, members of the membrane-associated guanylate kinase family (MAGUK), as well as 7H6,

cingulin, and symplekin (Fig.1) [Citi, 1993; Haskins et al., 1998; Mitic and Anderson, 1998; Balda and Matter, 1998]. Tight junctions and adherens junctions together form apical junctional complexes. Adherens junction-associated CMS proteins encompass symplekin, plakoglobin, α-catenin, β-catenin, vinculin, and undoubtably another host of known and unknown proteins, that interact with actin, as is the case also in tight junctions [Weiss et al., 1998] (Fig.1). Desmosomes constitute a third type of cell-cell junction, where adhesion is mediated through the desmosomal cadherins desmocollin and desmoglein and the CMS proteins desmoplakin and plakoglobin that are connected to interme-

diate filament type proteins [Green et al., 1998; Smith and Fuchs, 1998] (Fig. 1). As with cell-ECM adhesion complexes, cell-cell adhesion complexes interact with various kinases and phosphatases.

Although adhesion complexes are built with different components, they are connected to the same network of cytoskeletal filaments, their integrity depends on tyrosine kinase activity, and they share similar biochemical signal transducers [Yamada and Geiger, 1997]. This indicates that from one complex to another, the organization of cell adhesion complexes follows the same linear path including adhesion molecules linked to complexes of CMS proteins and kinases/phosphatases that regulate the induction of biochemical cascades and the organization of the cytoskeleton (Fig. 2), but it does not mean that these structures behave similarly. For instance, treatment with protein phosphatases disrupts FAs and the underlying cytoskeleton [Schneider et al., 1998], while increased tyrosine phosphorylation induces the redistribution of adherens and tight junction proteins [Collares-Buzato et al., 1998]. More specifically, adherens junction disassembly is due to in-

creased phosphorylation mediated through MAP kinase and PI 3-kinase pathways [Potempa and Ridley, 1998], whereas desmosome disassembly appears to be regulated by activation of PKC [Amar et al., 1998].

Cell adhesion complexes participate in the coordinated regulation of cell division, survival, and differentiation [Weaver et al., 1997; Bailey et al., 1998; Balda and Matter, 1998; Bissell, 1998; Clark et al., 1998; Perl et al., 1998; Sharma, 1998; Short et al., 1998]. This coordinated regulation of cell behavior is mediated by the integration of the linear paths of cell adhesion complexes through interconnection with other signal transduction cascades [Wang et al., 1998], and the link between actin and intermediate filament networks [Yang et al., 1996; Yamada and Geiger, 1997; Fuchs and Cleveland, 1998] (Fig. 2). This defines an integrated function for cell adhesion complexes, which is ruled by the equilibrium between the different adhesion structures. In addition, alternate construction and deconstruction of cell adhesion complexes has been shown to be critical for developmental programs and cell migration. In this case, modulation of cellular behavior is due

Fig. 2. Linear path and integrated functions of cell adhesion complexes. All cell adhesion complexes follow the same organizational path, which involves the recruitment of connective membrane skeleton (CMS) proteins upon cell-cell and cell-ECM interaction, and the induction of the formation of multiprotein complexes at the inner part of the cell membrane. These complexes include biochemical signal transducers such as kinases (Ki) and phosphatases (Ph) and cytoskeletal proteins and lead to the initiation of biochemical cascades and the reorganization of cytoskeletal fibers (A, actin; IF, intermediate filaments). This defines the linear path of cell adhesion complexes. The integrative function results from the influence of the various cell adhesion complexes on each other through the physical interconnection of cytoskeletal networks (linker) and the interactions between biochemical signaling pathways. The integrative function is ultimately responsible for the regulation of cytoplasmic and nuclear activities that determine the cell and tissue phenotype. Color plate on page 330.
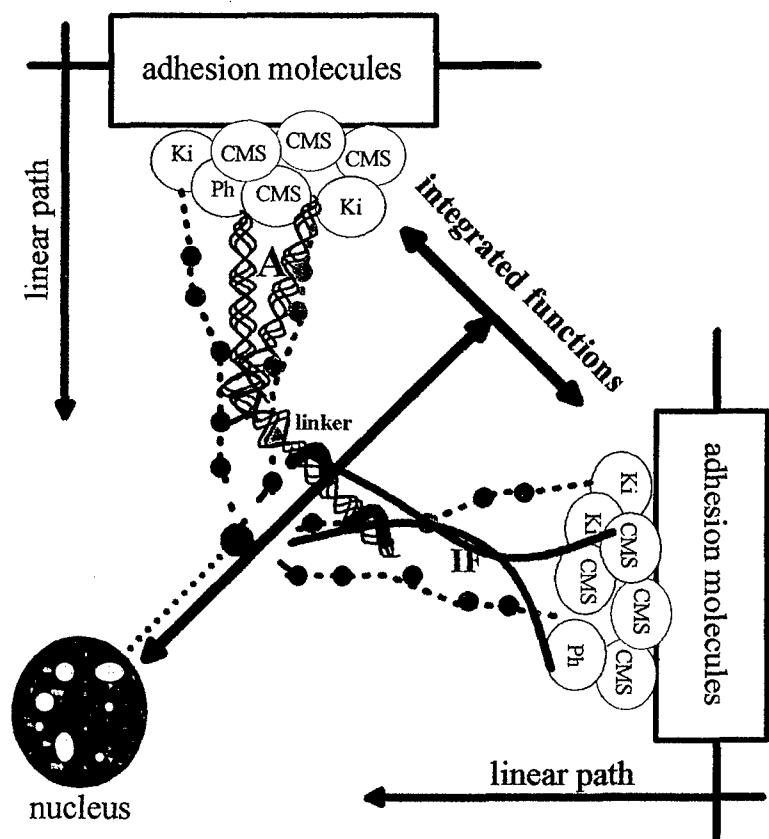
to the switch of dominant signaling pathways that results from equilibrium shifts and temporary delocalization of adhesion proteins and membrane skeleton proteins. However, if there is sustained imbalance, the equilibrium shift may also lead to the stimulation of tumor development [Sympson et al., 1995 and in preparation; Weaver et al., 1997; Thomasset et al., 1998; Efstathiou et al., 1998; Tselepis et al., 1998; Shinohara et al., 1998].

CONNECTIVE MEMBRANE SKELETON PROTEINS ALSO RESIDE IN THE NUCLEUS

The CMS proteins, β-catenin, plakoglobin, plakophilin 2, symplekin, ZO-1, and zyxin have all been observed in the nucleus. Logically, only the CMS proteins free from their interaction with adhesion complexes will translocate into the nucleus. The constitution of a free pool of CMS proteins could result from the expression of these proteins above the level necessary for the formation of adhesion complexes, as was the case after overexpression of exogenous plakoglobin in transfection experiments [Karnovsky and Klymkowsky, 1995]. However, CMS proteins are more likely to be observed in the nucleus when the formation of adhesion complexes is impaired, as it has been described in naturally occurring situations. Endogenous β-catenin has been found in tumor cell nuclei in which cell adhesion complexes were altered [Bailey et al., 1998]. Endogenous symplekin and plakophilin 2 were observed in the nucleus of cells that usually do not form adherens junctions or desmosomes [Mertens et al., 1996; Keon et al., 1996]. The presence of endogenous ZO-1 in the nucleus of epithelial cells was inversely correlated with the extent or maturity of tight junctions [Gottardi et al., 1996], and apical polarity in mammary acini (Lelièvre and Bissell, unpublished observations). The localization of these CMS proteins in cell adhesion complexes and in the nucleus is not mutually exclusive, as both locations have been simultaneously observed in many cases. This finding suggests that there may be an equilibrium between membrane skeleton and nuclear localization of the CMS proteins.

The creation of a free pool of CMS proteins via their release from existing cell adhesion complexes or through other mechanisms is not sufficient to explain how these proteins can enter the nucleus. The study of shuttling proteins has shown that the mechanisms of nuclear

translocation are highly regulated and it is conceivable that translocating CMS proteins may conform to the same mechanisms. It is known that proteins of >40 kD actively enter the nucleus by binding to the nucleopore proteins importins through a nuclear localization signal (NLS) and by translocating through an energy-dependent mechanism [Görlich and Mattaj, 1996]. While a putative NLS has been identified in the sequence of both ZO-1 and symplekin proteins [Gottardi et al,. 1996; Keon et al., 1996], the evidence that these NLS are functional is still lacking. If indeed they are functional, it will be worthwhile to analyze the possible nuclear localization of other NLS bearing CMS proteins (e.g., plectin) [Nikolic et al., 1996]. The presence of a functional NLS may not always be necessary for the nuclear translocation of CMS proteins. These proteins could "piggy-back" with other NLS-bearing molecules, as proposed for β-catenin, which travels as a complex with LEF-1 [Funayama et al., 1995; Behrens et al., 1996; Simcha et al., 1998]. NLS-free CMS proteins could also bind directly to the nuclear pore and translocate into the nucleus, as demonstrated recently for β-catenin [Fagotto et al., 1998]. It will be important to clearly classify those proteins that "piggy-back" from self-translocating CMS proteins. In the former case, the nuclear translocation is dependent not only on a free pool of CMS proteins, but also on the availability of their carrier.

Once CMS proteins are trapped in the nucleus, they may stay there until a signal induces their release from nuclear complexes and initiates their degradation. *De novo* expression of proteins would re-create the pool of CMS proteins in the cytoplasm. However, since the control of the nuclear translocation of CMS proteins is achieved by their release from interactions with cell membrane and cytoskeletal components, it is similarly possible that their release from interactions with nuclear partners would lead to their return to the cytoplasm. The study of protein shuttling has shown that proteins can slowly diffuse out of the nucleus without any specific signal [Schmidt-Zachmann et al., 1993], while a fast re-entry into the cytoplasm is regulated by pathways distinct from nuclear import mechanisms [Moroainu, 1998]. The rapid transit of nuclear proteins to the cytoplasm is mediated by nuclear export sequences (NES) via energy-dependent extrusion mechanisms [Wen et al., 1995; Richards et al.,

1996]. Thus far, only the CMS protein zyxin has been shown to possess a functional NES, and its traveling into and out of the nucleus has been observed in the course of antibody-injection experiments [Nix and Beckerle, 1997]. However, as with nuclear protein import, we could imagine that CMS proteins could also leave the nucleus by "piggy-backing." Bidirectional transit of zyxin could be regulated by temporarily masking the NES, and/or by the presence of a nuclear retention signal or cytoplasmic retention signal that would be responsible for the binding of the protein to nuclear or cytoplasmic components, as has been proposed for shuttling proteins [Wen et al., 1995; Nakielny and Dreyfus, 1996; Richards et al.,1996]. The participation of CMS proteins in the formation of adhesion complexes is an example of cytoplasmic retention.

DO NUCLEAR CMS PROTEINS ACT AS SIGNAL TRANSDUCERS?

The release of CMS proteins from cell adhesion complexes and their nuclear translocation are both highly regulated; thus, we can anticipate that the journey of CMS proteins to the nucleus is another important facet of signal transduction. Most of the CMS proteins bear sequences that link them to families of proteins known to participate in the mediation of signal transduction events, such as the armadillo family for β-catenin, plakoglobin, and plakophilin 2 [Peifer et al., 1994], and the MAGUK family for ZO-1 [Willot et al., 1993]. Zyxin harbors LIM domains known to participate in protein-protein interactions and that might interact with nucleic acids [Schmeichel and Beckerle, 1994, 1997; Nix and Beckerle, 1997; Beckerle, 1997]. The role of the nuclear translocation of CMS proteins in signal transduction is further supported by the fact that some CMS proteins are found in the nucleus of cell types that do not use these CMS proteins in the formation of their adhesion complexes [Mertens et al., 1996; Keon et al., 1996]. Interestingly, MAP kinases (erk1, erk2, and Nlk) known to be associated with cell adhesion complexes-mediated signaling have also been shown to travel to the nucleus. Their role as nuclear signal transducers is easier to grasp compared with CMS proteins because MAP kinases could drive the continuity of biochemical transduction cascades by exerting their kinase activity both in cytoplasmic and nucleoplasmic compartments [Sanghera et al.,

1992; Chen et al., 1992; Khokhlatchev et al., 1998; Brott et al., 1998]. Their translocation is regulated as shown for erk2, the nuclear import of which is promoted by phosphorylation-directed homodimerization [Khokhlatchev et al., 1998]. Another type of regulation of translocation has been described for MAPKAP kinase2, which is exported to the cytoplasm upon stress induction and also harbors an NLS [Engel et al., 1998]. Potential nuclear targets of MAP kinases include Rb [Taieb et al., 1998], histone H3, c-Fos, c-Jun, and transcription factors phosphorylated in response to growth stimuli [Chen et al., 1992]. Nuclear MAP kinases have also been proposed to participate in the regulation of insulin gene transcription [Benes et al.,1998].

The best evidence for a meaningful function for nuclear CMS proteins as signal transducers would require the characterization of the cellular behaviors associated with their presence in the cell nucleus and the identification of their nuclear binding partners. This goal has been achieved partly for nuclear β-catenin, a component of the Wnt signaling pathway, which has been implicated in the induction of embryonic axis in *Xenopus* and the regulation of gene expression in higher organisms via the formation of a complex with transcription factor LEF-1 and DNA [McCrea et al., 1993; Funayama et al., 1995; Gumbiner, 1995; Behrens et al., 1996; Papkoff et al., 1996; Brannon et al., 1997; Larabell et al., 1997; Miller and Moon, 1997]. More specifically, LEF-1-β catenin complexes have been shown to bind to the 5′ end of the E-cadherin gene [Huber et al., 1996]. This demonstrate that nuclear CMS proteins may act as repressors or activators of transcription.

Obviously, at this point there are more questions than answers for the mode of action of CMS proteins. Before answers are forthcoming, a number of technical points need to be clarified. The choice of immunostaining conditions appears to be critical for observing CMS proteins in the nucleus. Thus, caution has been advised in the use of permeabilization agents, and in some cases, a special protection buffer had to be used [Keon et al, 1996; Gottardi et al.,1996]. A careful monitoring of immunostaining conditions may allow the observation of other types of CMS proteins in the nucleus. The fact that, in a number of cases, CMS proteins located in the nucleus have been shown to be sensitive to the use of permeabilization agents indicates that although these proteins are asso-

ciated with the cytoskeleton in the cytoplasm, they are not likely to be associated with the nucleoskeleton or nuclear matrix in the nucleus. The only CMS protein so far described to be a nuclear matrix protein, because it stays associated with cell remnants following DNA removal under high salt conditions, is protein band 4.1. This protein is associated with the membrane skeleton in red blood cells [Matsuzaki et al.,1985] and other cells [Granger and Lazarides, 1984; Leto et al., 1986], and has been reported to be present in the nuclei of numerous cell types [Coreas, 1991; de Carcer et al., 1995; Krauss et al., 1997; Lallena and Coreas, 1997]. However, because some of the isoforms found in the nucleus have been shown to be the product of alternative splicing, this undermines a possible role of protein 4.1 in direct signal transduction between the cell membrane and the nucleus [Tang et al., 1988; Luque et al., 1998; Lallena et al., 1998]. A demonstration that the same isoform of protein 4.1 shuttles between the cytoplasm and nucleus requires further experiments and proof.

## A CURRENT PERSPECTIVE ON THE MECHANISMS OF COMMUNICATION

The dual function of the CMS proteins located at the cell membrane that act both as initiators of biochemical cascades and organizers of the cytoskeleton, plays a pivotal role in cell adhesion-mediated extracellular information. Once released from adhesion complexes, CMS proteins are available for interaction with other cellular components and for possible nuclear translocation. Thus, they become powerful signal transducers by directly transferring information from the source, a specific type of cell adhesion complex formed as a response to microenvironmental cues, to nuclear targets. Whether additional intermediate steps are needed is an open question. An interesting hypothesis for a new level of regulation of directed-protein localization has been proposed. In this model, proteins would reach specific cell membrane or nuclear substructures via a defined targeting sequence [Wu et al., 1998; Cardoso and Leonhardt, 1998]. This model seems especially tempting in the case of shuttling proteins that may bear targeting sequences for substructures in both cytoplasmic and nuclear compartments. Specific sequences of β-catenin are responsible for its binding to E-cadherin and

α-catenin in the cytoplasm and although its *arm* sequence has been shown to be necessary for nuclear targeting, its nuclear substructural destination is not yet known [Behrens et al.,1996].

CMS proteins can be considered as active mediators of the dynamic reciprocity between the microenvironment and the cells. We suggest that there is a bidirectional flow of information between the microenvironment and the nucleus, part of which depends on a molecular equilibrium defined by the binding of CMS proteins to their various partners. This concept is well illustrated by the data generated for β-catenin (Fig. 3). Free cytoplasmic β-catenin, the presence of which depends on its association with other adherens junction molecules and cell membrane receptors, has to override the APC-regulated degradation mechanism [Munemitsu et al.,1995] before going to the nucleus. The number of free β-catenin molecules also has to exceed the amount required to form complexes with free ZO-1 that were shown to participate in the formation of tight junctions [Rajasekaran et al., 1996].

Displacement of individual molecular equilibria resulting in the accumulation of CMS proteins in the nucleus, subsequently influences another level of equilibrium that exists between distant cellular compartments (e.g., membrane skeleton and nucleus) (Fig. 4). A prolonged shift in the compartmental equilibrium may ultimately lead to the development of aberrant cellular behavior, including malignancy. We have recently shown that it is possible to reestablish the equilibrium and to revert the malignant phenotype by correcting the levels and the signaling of cell adhesion components [Weaver et al., 1997], as well as other cell surface receptors [Wang et al., 1998]. Thus, an "oncogene" [β-catenin—Peifer, 1997] or tumor suppressor [ZO-1-Willot et al., 1993] function could be linked to the relative localization of CMS proteins, depending on whether they are in the nuclear or in the membrane skeleton compartment. The cytoplasmic localization of c-abl kinase has been associated with the expression of the malignant phenotype [Van Etten et al., 1989; Sawyers 1992], whereas in nontumor cells, its nuclear localization has been demonstrated to be essential for the inhibition of cell growth [Sawyers et al., 1994]. Interestingly, c-abl is primarily considered a nuclear protein [Van Etten et al., 1989]. It binds to DNA

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Fig. 3. Molecular equilibrium. CMS proteins are engaged in a number of interactions with different cellular components. The relative abundance of the binding partners of CMS proteins regulates the molecular equilibrium, which in turn affects cellular function. For example, β-catenin can interact with cadherins (*light blue*), ZO-1 (*green*), APC (*dark blue*), EGF receptor (EGF-R), and erb-2 at the cell membrane. Moreover, cadherin/catenin, EGF-R, β-1-integrin and α6-β4 integrins interact with each other. β-Catenin binding with transcription factor LEF-1(*brown*) and its translocation to the nucleus, depends on the existence of a free pool of β-catenin; it may also be influenced by the amount of LEF-1 available. Other factors may contribute to the shift of the molecular equilibrium, like the interaction of plakoglobin with β-catenin partners, and Wnt-1 expression which influences the degradation process associated with the formation of APC/β-catenin complexes. The presence GSK3 β and Axin that interact with APC/β-catenin complexes may also influence the molecular equilibrium. The return of β-catenin from the nucleus (dotted arrow) to interact with cell membrane binding partners, upon reception of specific signals, remains to be demonstrated. **Color plate on page 330.**
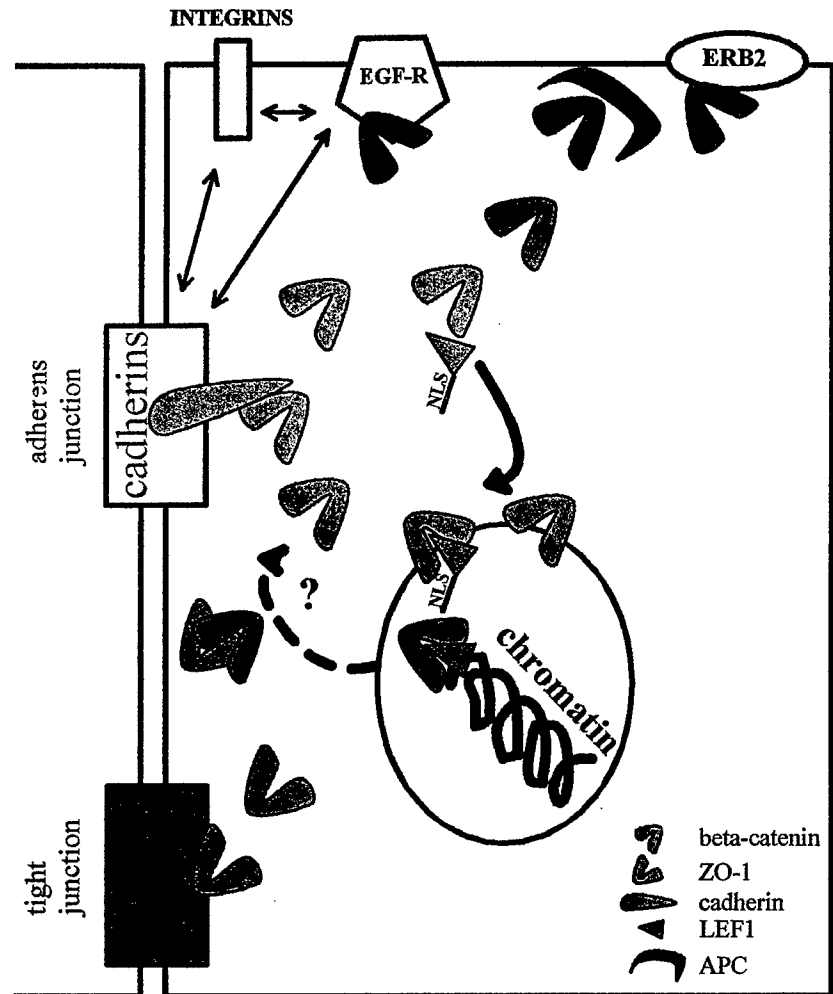
and RNA polymerase II [Kipreos and Wang, 1992; Rajasekaran et al., 1996]. Moreover, Rb protein has been reported to bind c-abl during G1 phase and to inhibit its kinase activity [Welch and Wang, 1993]. Nevertheless, c-abl does not require Rb to exert its growth suppressive activity [Sawyers et al., 1994]. c-abl harbors NLSs and a functional NES and has been shown to shuttle between the nucleus and the cytoplasm [Lewis et al., 1996; Taagepera et al., 1998]. The rate of nuclear import and export of c-abl is regulated by adhesion to the ECM, which also regulates c-abl tyrosine kinase activity [Lewis et al., 1996]. Since c-abl activation requires cell adhesion, and adhesion to ECM recruits c-abl to early focal adhesions coincident with export of the protein from the nucleus, and finally the active nuclear c-abl seems to originate from the cytoplasmic pool activated by adhesion, it has been proposed that c-abl is an important mediator of integrin signals to the nucleus [Lewis et al., 1996]. It has also been

proposed recently that CMS or adhesion plaque proteins (e.g., plakophilins, β-catenin, plakoglobin, symplekin) may be genuine nuclear proteins that could be recruited to the membrane skeleton to participate in the assembly of cell adhesion complexes during cell differentiation [Keon et al., 1996]. This seductive hypothesis will require more evidence starting with the elucidation of the role played by CMS proteins in the nucleus. CMS proteins that translocate to the nucleus may also be shuttling proteins, as suggested for zyxin [Nix and Beckerle, 1997].

Shuttling proteins are defined as proteins that continuously travel from the cytoplasm to the nucleus [Schmidt-Zachmann et al., 1993]. Although not always demonstrated, it is understood that shuttling must be associated with the transport of information. If primary localization sites of shuttling proteins are in the cytoplasm, as determined by the visualization of supramolecular organizations due to the cytoplasmic concentration of shuttling protein-
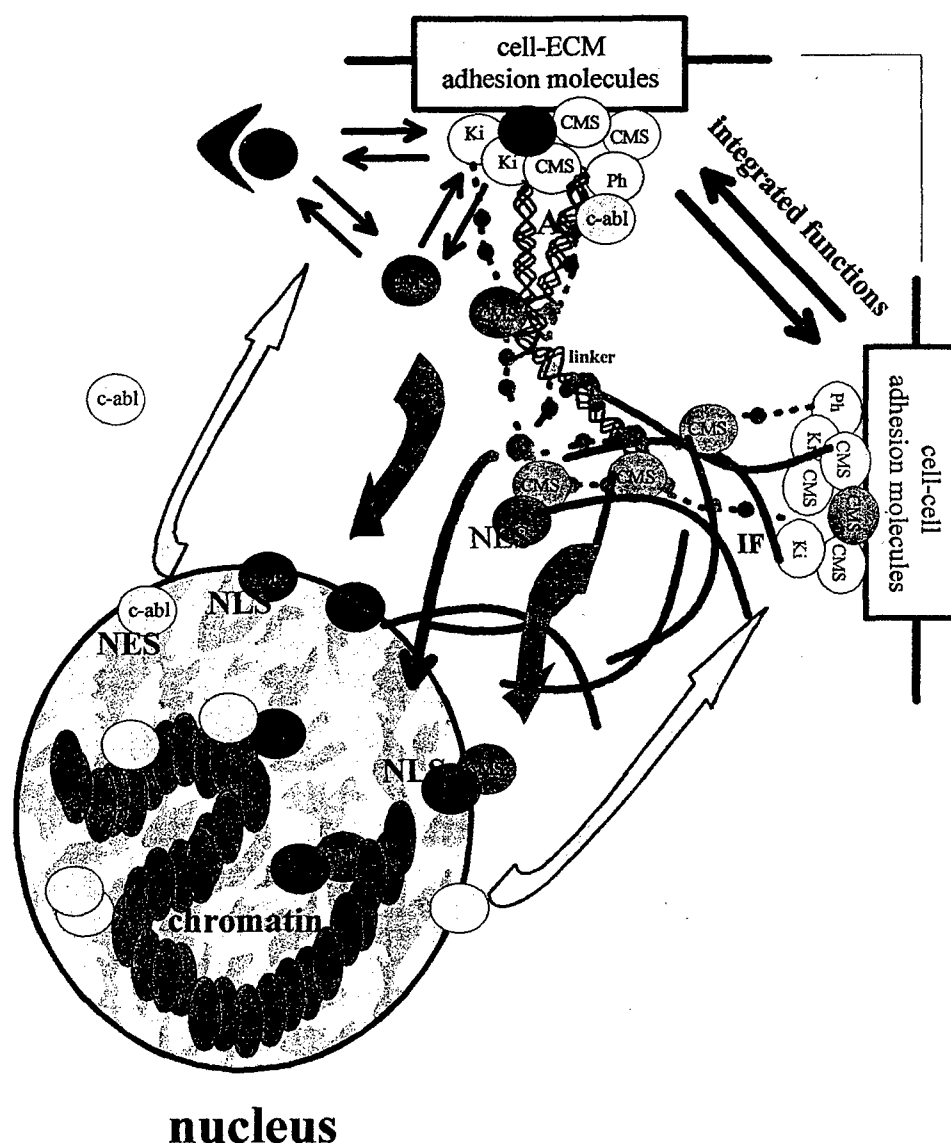
cell-ECM
adhesion molecules

Ki

CMS

CMS

Ki CMS

Ph

c-abl

integrated functions

c-abl

linker

cell-cell
adhesion molecules

Ph

CMS

CMS

CMS

CMS

CMS

CMS

Ki

CMS

NES

NLS

IF

c-abl

NLS

NES

NLS

chromatin

nucleus

Fig. 4. Compartmental equilibrium and dynamic reciprocity. Nuclear translocation of CMS proteins (*red* and *orange*) modifies the balance of these proteins between the membrane skeleton compartment and the nuclear compartment and shifts their molecular equilibrium toward an increase of interactions with nuclear targets. Translocating CMS proteins can be considered as structural signal transducers that act as mediators of cell-cell and cell-ECM signaling, along with biochemical cascades (*light green*) possibly superimposed on signaling via tension generated through actin (A) and intermediate filament (IF) networks. CMS proteins could translocate into the nucleus on their own (*red*) and bind to nuclear proteins (*yellow*), or they could travel with a carrier (*dark green*). The transfer of information from the nucleus to the cell membrane includes the synthesis of membrane skeleton, cell membrane, and ECM components, it may also involve feedback reactions to generated tensional force (not represented). The translocation of nuclear CMS proteins back to the membrane skeleton may also participate in inside/out signaling. Similarly, proteins primarily located in the nucleus could travel to the cell membrane (*yellow*), as shown with c-abl. We propose that nuclear structural proteins involved in supramolecular organization of the nucleus also may travel to the cell membrane. The balance between these interactive signaling pathways represents the dynamic reciprocity that governs cellular and tissue behavior. **Color plate on page 331.**

binding partners or the masking/unmasking of specific targeting sequences, a very rapid translocation in and out of the nucleus will result in immunostaining seen almost exclusively in the cytoplasm. The same line of reasoning is true for a primary localization site in the nucleus.

Many types of supramolecular organization have been described in the nucleus, including transcription sites, speckles, coiled bodies, and PML bodies [Nickerson et al., 1995]. Specific types of supramolecular organization in the nucleus have also been described to occur in

association with tissue-like morphogenesis [Lelièvre et al., 1998]. The shuttling of components found primarily in the nucleus has been described for snRNAs and RNA-binding proteins, as well as for proteins participating in nuclear import, heat shock proteins, and nucleolar proteins [Schmidt-Zachmann et al., 1993; Görlich and Mattaj, 1996], and the shuttling of molecules from sites predominantly located in the nucleus to the cytoplasm has been suggested to participate in the regulation of nuclear functions.

Sooner or later, we must put a plan together as to how parts are integrated to bring about homeostasis. Recent data demonstrating the coupling of integrins and EGF receptor pathways in epithelial cells cultured three-dimensionally in the presence of a reconstituted basement membrane, as opposed to monolayer culture [Wang et al., 1998], as well as the demonstration of movement of specific mRNAs to focal adhesion complexes [Chicurel et al., 1998], point to the intimate relationship between positional and functional information. To complete the dynamic reciprocity scheme, we anticipate that, as with the behavior of CMS proteins, more resident structural components of the nucleus may travel in the opposite direction to the cell membrane and hence act as nuclear signal transducers.

ACKNOWLEDGMENTS

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Tissue Structure, Nuclear Organization, and Gene Expression in Normal and Malignant Breast[1]

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Abstract

Because every cell within the body has the same genetic information, a significant problem in biology is to understand how cells within a tissue express genes selectively. A sophisticated network of physical and biochemical signals converge in a highly orchestrated manner to bring about the exquisite regulation that governs gene expression in diverse tissues. Thus, the ultimate decision of a cell to proliferate, express tissue-specific genes, or apoptose must be a coordinated response to its adhesive, growth factor, and hormonal milieu. The unifying hypothesis examined in this overview is that the unit of function in higher organisms is neither the genome nor the cell alone but the complex, three-dimensional tissue. This is because there are bidirectional connections between the components of the cellular microenvironment (growth factors, hormones, and extracellular matrix) and the nucleus. These connections are made via membrane-bound receptors and transmitted to the nucleus, where the signals result in modifications to the nuclear matrix and chromatin structure and lead to selective gene expression. Thus, cells need to be studied "in context", *i.e.,* within a proper tissue structure, if one is to understand the bidirectional pathways that connect the cellular microenvironment and the genome.

In the last decades, we have used well-characterized human and mouse mammary cell lines in "designer microenvironments" to create an appropriate context to study tissue-specific gene expression. The use of a three-dimensional culture assay, developed with reconstituted basement membrane, has allowed us to distinguish normal and malignant human breast cells easily and rapidly. Whereas normal cells become growth arrested and form organized "acini," tumor cells continue to grow, pile up, and in general fail to respond to extracellular matrix and microenvironmental cues. By correcting the extracellular matrix-receptor (integrin) signaling and balance, we have been able to revert the malignant phenotype when a human breast tumor cell is cultured in, or on, a basement membrane. Most recently, we have shown that whereas β1 integrin and epidermal growth factor receptor signal transduction pathways are integrated reciprocally in three-dimensional cultures, on tissue culture plastic (two-dimensional monolayers), these are not coordinated. Finally, we have demonstrated that, rather than passively reflecting changes in gene expression, nuclear organization itself can modulate cellular and tissue phenotype. We conclude that the structure of the tissue is dominant over the genome, and that we may need a new paradigm for how epithelial-specific genes are regulated *in vivo.* We also argue that unless the structure of the tissue is critically altered, malignancy will not progress, even in the presence of multiple chromosomal mutations.

Introduction

"Science is built up with facts, as a house is with stones. But a collection of facts is no more science than a heap of stones is a house."–Jules Henri Poincaré (1854–1912)

In adult organisms, cells must maintain the program of regulated gene expression that is instituted during development. What are the genomic rules that allow this program of gene expression to be selective? Even after the cells have arrived at their "destinations" within the tissues, they radically alter their patterns of gene expression, both quantitatively and qualitatively, as a result of systemic (hormones, chemokines, and environmental insults) and microenvironmental (cell-ECM, cell-cell, local growth factors, local injury, and others) signals. This is observed most dramatically when cells are isolated and cultured outside of the organism, most notably on tissue culture plastic (as 2D[3] monolayers; 1). Along with loss of tissue-specific gene expression, the most striking change is in cellular and nuclear organization and architecture (2).

One of us proposed almost two decades ago that in addition to growth factors and hormones, ECM that surrounds tissues *in vivo* contains signaling molecules that are responsible for maintenance of tissue form and function (3), and furthermore, that there may be both mechanical and biochemical connections between the ECM and the nuclear skeleton, leading to changes in chromatin structure and gene expression. The combined work of many investigators, including our own, has confirmed the significance of the ECM in every tissue examined (reviewed in Refs. 4–6). In addition, the discovery of the ECM receptors, the most important family of which is the integrins (reviewed in Refs. 7 and 8), has elucidated a mechanism by which ECM signaling could be achieved across the cellular membrane.

In parallel, the work of structural and molecular biologists has unraveled important new information about the structure of the nucleus and the chromatin. The higher order structure of eukaryotic chromosomes consists of independent loop domains that are separated from each other by the attachment of specialized genomic sequences (matrix attachment regions) onto the NM (reviewed in Refs. 9–11). This organization is important not only to compact DNA but also for various functions involving DNA. Matrix attachment regions have been shown to be essential for demethylation of chromatin domains (12) and chromatin accessibility (13), processes directly implicated in regulating gene expression.

The compaction of eukaryotic DNA into chromatin is thought to establish a specific pattern of gene expression. Heterochromatin, defined cytogenetically as regions of the genome that remain condensed throughout the cell cycle, is known to remain transcriptionally silent. Translocation of a euchromatic region of the genome to a site adjacent to heterochromatin often yields variable silencing of the translocated genes, as exemplified by the process of position effect variegation of the eye color gene brown, in *Drosophila* (14). Other examples of chromatin packaging associated with long-term transcriptional repression is the transcriptional silencing observed at the mating

type loci or at telomeres in yeast (15, 16). From these and many other studies, histones are emerging as substrates for activities controlling transcription.

Histone acetylation is a crucial and evolutionary conserved mechanism, allowing chromatin reorganization because it loosens up histone-DNA interactions by neutralization of the net charge of histone tails and disruption of nucleosome-nucleosome interactions (17, 18). Acetylated lysine residues in the NH_2-terminal tail domains of nucleosomal histones allow us to distinguish euchromatin from heterochromatin, where silent heterochromatin is hypoacetylated (19). The level of histone acetylation is determined by an equilibrium between histone acetyltransferases and deacetylases. The opportunity for chromatin structure to be precisely modulated through highly regulated reversible mechanisms offers the possibility of transcriptional silencing or activation by this mechanism.

Synopsis of Previous Results and the Relevant Literature

The above brief summary points to a vast, and as yet only minimally understood, area of how organization of chromatin and nuclear architecture may be regulated within specific tissues.

The following provides an abbreviated summary focused largely, but not exclusively, on the work of our laboratory and those of our collaborators to provide a background for our working hypothesis on the relation of tissue structure and normal and malignant behavior (Fig. 1):

(a) There is evidence that even "universal" regulatory pathways,

such as apoptosis, are clearly tissue specific, e.g., radiation-induced apoptosis in vivo is p53 dependent in thymus and sphingomyelinase dependent in endothelium (20).

(b) It is now well established that ECM and its receptors critically affect tissue-specific structure and gene expression in all tissues; the mammary gland and MECs provide a well-studied example (reviewed in Ref. 5; Fig. 1).

(c) ECM affects both cellular "shape" and biochemical signaling (21, 22). Cytoskeletal shape-induced signaling is sufficient to modulate genes (such as lactoferrin; Refs. 22 and 23) and differential splicing of at least one protein examined thus far (band 4.1; 24).

(d) The magnitude of the ability of the ECM to influence gene transcription is exemplified by its ability to regulate the activity of some of the most potent transcriptional activators in eukaryotic cells, the viral enhancers (25, 26).

(e) Promoter sequences of both mammary-specific genes and growth factors contain ECM-responsive elements. β-Casein gene promoter contains a 161-bp enhancer (BCE-1; see Fig. 1) that is induced strongly (15–150-fold) by ECM (27, 28). The transforming growth factor β promoter, on the other hand, is completely suppressed by ECM (29).

(f) The ECM-response element of the β-casein gene (BCE-1) is active only within a chromatin context (this is true also for viral enhancers mentioned above). Furthermore, it now appears that contact with ECM alters the histone acetylation/deacetylation of the enhancer (30).
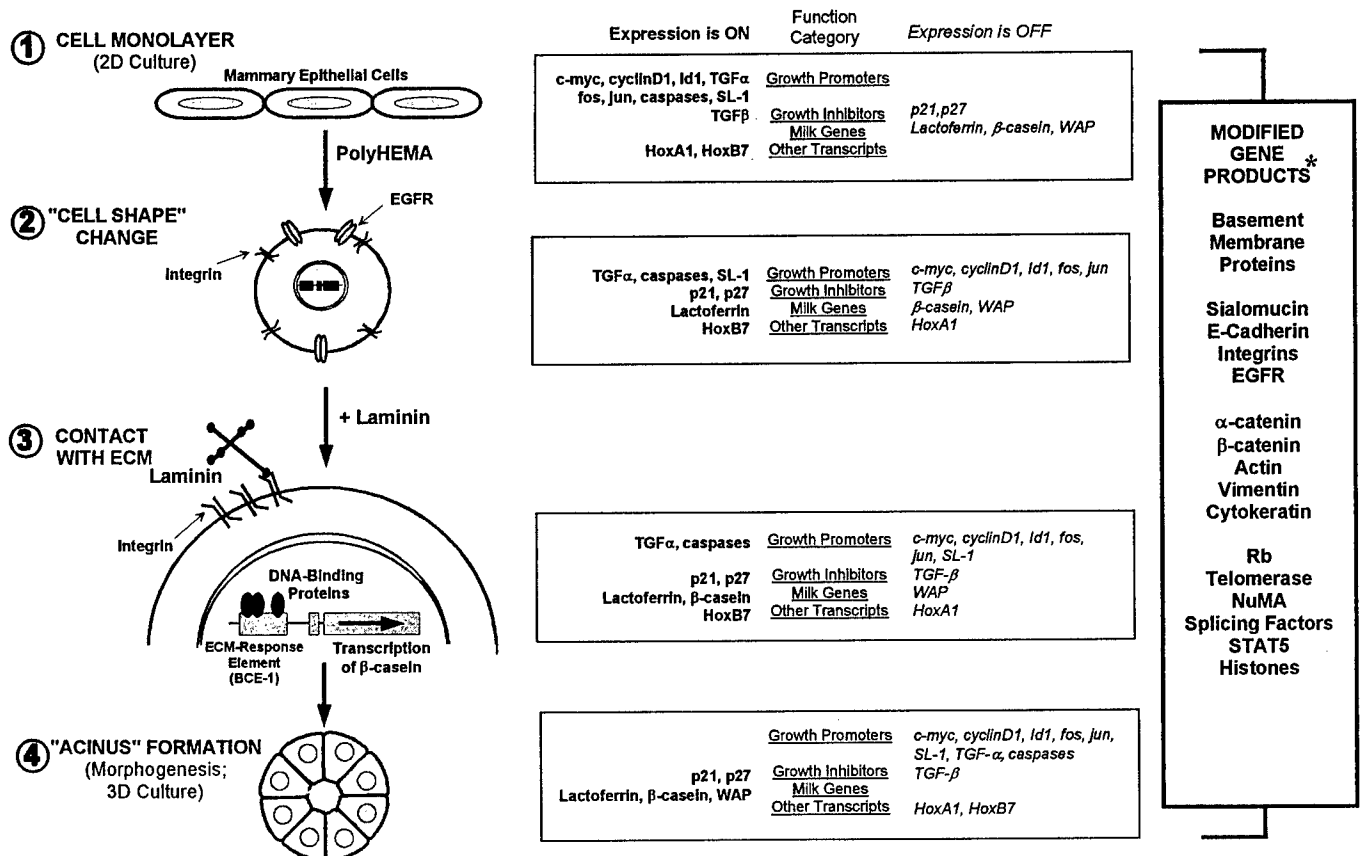

Fig. 1. Examples of gene products altered by contact with the ECM and changes in cellular structure. Studies of MECs cultured in the context of a variety of "designer microenvironments" have demonstrated that cells display distinct behaviors in response to changes in shape and ECM composition. In these cultures the inert substrate, polyHEMA, was used to model cell shape change by itself, whereas purified laminin was used as a ligand that stimulates integrin-dependent signaling; in vivo, the ECM is responsible for both of these steps. Using this approach, we have demonstrated that, as cells transition from 2D to 3D culture, the expression of distinct cassettes of genes is reciprocally modulated (i.e., many growth promoters are down-regulated, whereas growth inhibitors and milk genes are up-regulated). Other gene products (see *), although appearing to be "constitutively" expressed, are modified with respect to localization, levels, splicing patterns, or phosphorylation patterns. All of these events are precisely orchestrated to enable tissue differentiation and morphogenesis. The sketch in the figure is modified from Roskelley et al. (5).
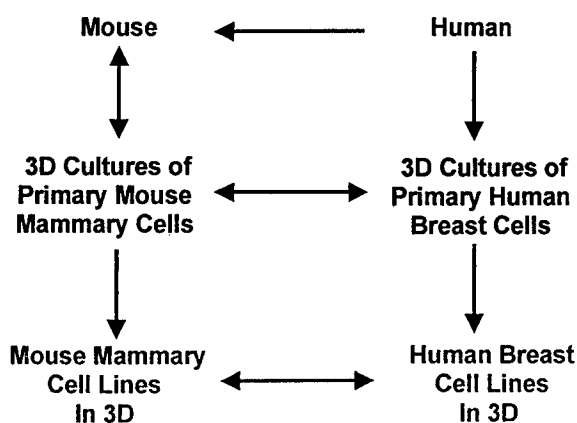
Fig. 2. Mouse and human mammary epithelial cell models provide complementary systems for the study of breast function. Although mouse and human mammary tissue vary somewhat with respect to overall organization, the double-layered structure of the branching ducts and ductules is found in both organisms. In light of these fundamental similarities, it is not surprising that human and mouse epithelial cells display similar behaviors in 3D basement membrane cultures; both cell types undergo morphogenesis to form spherical alveolar structures that are similar to acini *in vivo*. This observation bridges the gap between studies in humans and mice and justifies the use of the mouse to model aspects of normal and malignant human breast function.

(*g*) ECM and ECM-degrading enzymes have been shown to be central regulators of growth, apoptosis, branching morphogenesis, and epithelial to mesenchymal conversion and may play a role in mammary tumor induction and invasion (31–36; briefly reviewed in Ref. 37).

(*h*) Nuclear localization and half-life of important genes, such as *Abl* and *p53,* change dramatically as a result of ECM ligation and changes in cellular shape (38, 39).

(*i*) ECM regulates transcription factors such as Id-1 (40) and cell surface receptors such as EGFR (Ref. 41 and see below), which when overexpressed will override the ECM-induced morphogenesis (*i.e.,* "acinus" formation) and push the cells back into the cell cycle.

(*j*) By manipulating the cell surface, we can "revert" a disorganized and malignant human breast cell (HMT-3522, see below) to a quiescent, practically normal phenotype using inhibitory antibodies to β1 integrin (42). Phenotypic reversion is associated with dramatic changes in levels of cyclin D1, p21, and other growth parameters (such as the retinoblastoma susceptibility gene, *Rb*)[4] both in culture and *in vivo* without a change in tumor genotype.

Modeling Normal and Malignant Mammary Gland in Culture

To address the question of how the bidirectional flow of information sets up the 3D structure of a tissue and how this in turn governs selective gene expression, we have used mammary epithelial cells and the mammary gland itself as our central model. Why the breast?

(*a*) It is one of the few tissues that can be induced to undergo dramatic shifts in structure and function as a result of extracellular cues, even during the adult life of the organism.

(*b*) Despite the inherent complexity in organization and function of any tissue, it is relatively simple (compared, for example, to the brain, lung, or liver) and thus can be modeled in culture.

(*c*) The breast is a highly sensitive target of radiation and environmental insults, and breast cancer is a devastating disease in need of diagnosis, prognosis, cure, and prevention.

(*d*) Breast cancer cells provide examples of loss of structure and altered gene expression and thus could be used as natural "mutants" for comparisons of genotype and phenotype.

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[4] Unpublished data.

(*e*) A number of mouse and human mammary epithelial cell models exist (43, 44). Together they provide tractable and complementary systems for the study of mammary gland function and tumorigenicity (Fig. 2). For example, results obtained from studies of mouse and human cells in culture can be verified by transplanting these cells back into mice. Comparisons between the behavior of the transplanted cells, both human and mouse, and that of the same cells in culture provide useful information that ultimately contributes to the understanding of breast functions *in vivo* in humans.

The HMT-3522 Breast Tumor Progression Series

One recently described human MEC model, the HMT-3522 series, has proven to be particularly useful in the study of human breast cancer progression (45). The HMT-3522 cell series originated from a purified luminal epithelial cell population recovered from a breast biopsy of a woman with fibrocystic breast disease. These cells, collectively referred to as S1, have been cultured under chemically defined conditions for >500 passages (46). Despite the fact that S1 cells from later passages are notably aneuploid and carried a mutation in the *p53* gene, none of these cells have yet given rise to tumors in nude mice. Because growth autonomy has been cited as a prerequisite for malignant conversion, Briand and co-workers reasoned that removal of EGF from the HMT-3522 medium might eventually induce malignant transformation in S1 cells. Thus, to generate a tumorigenic HMT-3522 cell species, S1 cells (at passage 118) were grown in the absence of EGF. After ~120 passages, these EGF-free cultures gave rise to tumors in nude mice; cells cultured from these tumors were called T4-2 cells (47).

Although cells of the HMT-3522 series are hard to distinguish when cultured on plastic as 2D monolayers, phenotypic differences between the various HMT-3522 cell populations can be readily detected when these cells are cultured in a 3D reconstituted basement membrane (Fig. 3; Refs. 42 and 48). In this system, nonmalignant S1 cells form phenotypically normal structures reminiscent of terminal duct lobular units *in situ,* whereas their premalignant and tumorigenic counterparts form disorganized, continuously growing colonies. The following discussion will describe studies of S1 (passage 50) and T4-2 cells, exclusively.

Reversion of the Malignant Phenotype by β1 Integrin Inhibitory Antibody

Recent studies, using both *in vivo* and culture models, have demonstrated a role for cell-ECM receptors, or integrins, in human breast tumor progression (42, 48–51). Integrins are a class of heterodimeric transmembrane receptor proteins that mediate cell anchorage, influ-

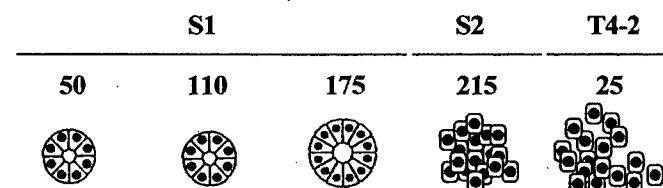| S1			S2	T4-2
50	110	175	215	25

Fig. 3. Cells from the HMT-3522 human breast cancer progression series exhibit characteristic phenotypes in 3D basement membrane cultures. The HMT-3522 progression series was originally derived from purified epithelial cells recovered from a woman with fibrocystic disease. These nonmalignant cells were grown under defined conditions for more than 10 years, during which time they were assayed for tumorigenic behavior. When cultured inside of a 3D reconstituted basement membrane, the nonmalignant S1 cells at passages 50 and 110 form endogenous basement membrane and exhibit a functionally normal phenotype (a single-layered acinus), which is comparable to acini derived from reduction mammoplasty. Later passage S1 cells, S1-175, also form acinar structures, but the structures are generally larger than those formed by S1-50 cells. Premalignant S2 cells and T4-2 tumor cells form large, irregular colonies in 3D basement membrane cultures. Thus, the use of a 3D basement membrane assays allow for phenotypic classification of these different cell types, which includes a phenotype for "premalignant."

ence cell shape, and propagate intracellular signals similar to those observed during growth factor receptor activation (7, 8, 52). Given that cell-ECM interactions are dramatically disrupted in breast cancer tissue, we compared the integrin profile of the "normal" nontumorigenic S1 cells to that displayed by tumorigenic T4-2 cells (42). The results showed that the levels of both total and surface-expressed β1 integrin proteins were significantly higher in the T4-2 cells than in the nontumorigenic S1 cells. To test the possibility that inappropriate integrin expression and activation contributes to the tumorigenic behavior of the T4-2 cells, a β1 integrin function-blocking antibody (monoclonal antibody AIIB2) was added to the 3D cultures of T4-2 cells. Upon β1 integrin inhibition, T4-2 cells underwent a striking morphological and functional normalization, characterized by reformation of acini with reassembled basement membranes, normal cell-cell contacts and cellular polarity, and a reorganized actin cytoskeleton (Fig. 4A). Furthermore, these "reverted" cells became growth arrested and were found to exhibit reduced tumorigenicity in nude mice (42). The program of pleiotropic changes that occur in T4-2 cells as a result of treatment with β1 integrin inhibitory antibody are summarized in Fig. 4B. These findings provide an independent demonstration that integrins, by virtue of their ability to sense and respond to cues from the ECM, can have profound effects on cellular perception of the microenvironment and therefore ultimately on cellular behavior. Furthermore, cells can harbor a myriad of chromosomal mutations, as is the case with T4-2 cells (determined by comparative genomic hybridization; not shown), but as long as the cell detects an appropriate cellular microenvironment that allows a cell to adopt a normal structure, the cell will display a normal phenotype. Our conclusions are supported by a recent report by Deng *et al.* (53), demonstrating that, although breast tissue found adjacent to neoplastic lesions display normal cellular morphologies *in situ*, cells within these lesions often harbor chromosomal rearrangements (*i.e.*, loss of heterozygosity), identical to some of the mutations found in the adjacent tumor.
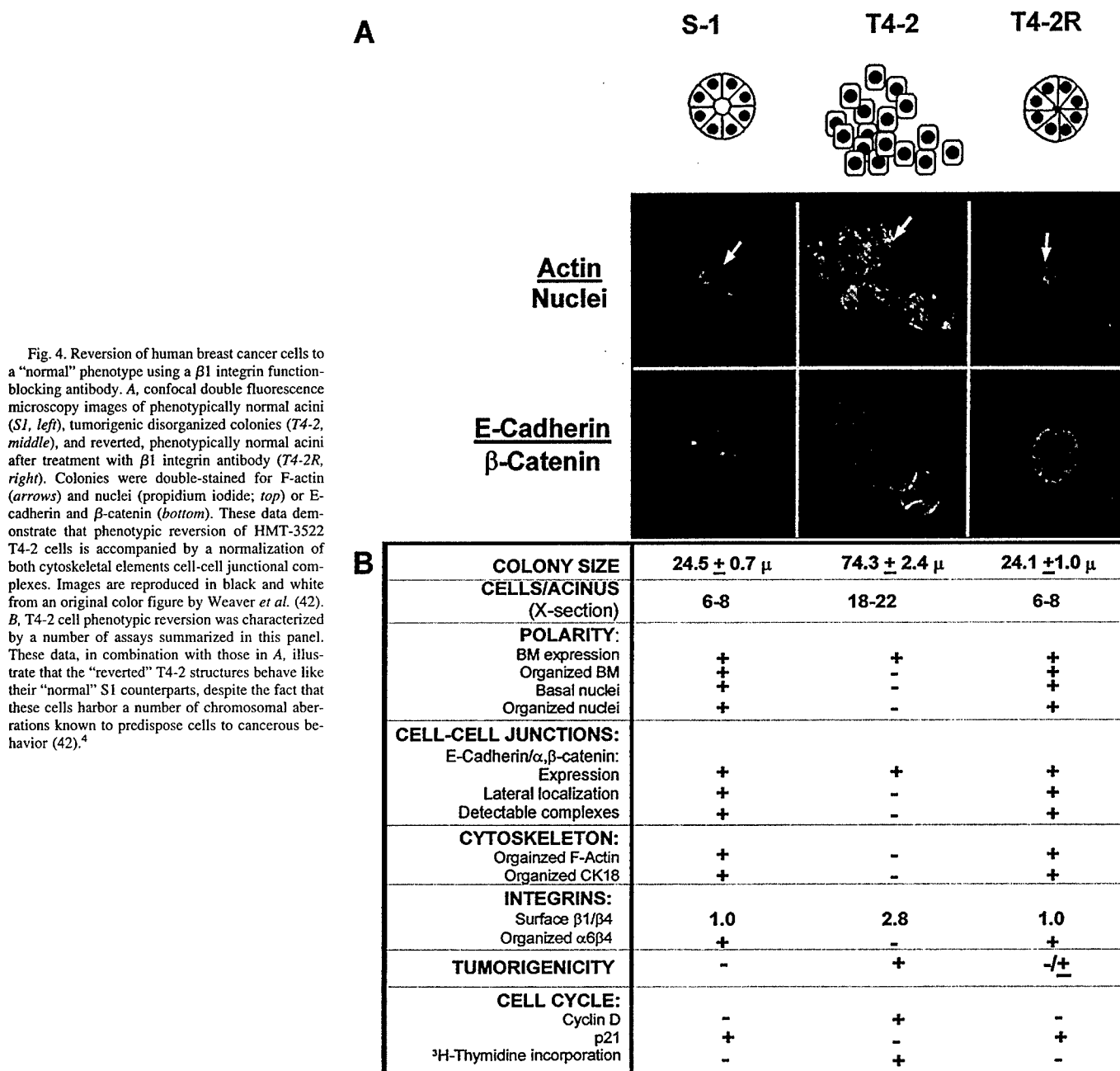

Fig. 4. Reversion of human breast cancer cells to a "normal" phenotype using a β1 integrin function-blocking antibody. A, confocal double fluorescence microscopy images of phenotypically normal acini (*S1, left*), tumorigenic disorganized colonies (*T4-2, middle*), and reverted, phenotypically normal acini after treatment with β1 integrin antibody (*T4-2R, right*). Colonies were double-stained for F-actin (*arrows*) and nuclei (propidium iodide; *top*) or E-cadherin and β-catenin (*bottom*). These data demonstrate that phenotypic reversion of HMT-3522 T4-2 cells is accompanied by a normalization of both cytoskeletal elements cell-cell junctional complexes. Images are reproduced in black and white from an original color figure by Weaver *et al.* (42). B, T4-2 cell phenotypic reversion was characterized by a number of assays summarized in this panel. These data, in combination with those in A, illustrate that the "reverted" T4-2 structures behave like their "normal" S1 counterparts, despite the fact that these cells harbor a number of chromosomal aberrations known to predispose cells to cancerous behavior (42).[4]

	S-1	T4-2	T4-2R
COLONY SIZE	24.5 ± 0.7 μ	74.3 ± 2.4 μ	24.1 ±1.0 μ
CELLS/ACINUS (X-section)	6-8	18-22	6-8
POLARITY:			
BM expression	+	+	+
Organized BM	+	-	+
Basal nuclei	+	-	+
Organized nuclei	+	-	+
CELL-CELL JUNCTIONS:			
E-Cadherin/α,β-catenin:			
Expression	+	+	+
Lateral localization	+	-	+
Detectable complexes	+	-	+
CYTOSKELETON:			
Organized F-Actin	+	-	+
Organized CK18	+	-	+
INTEGRINS:			
Surface β1/β4	1.0	2.8	1.0
Organized α6β4	+	-	+
TUMORIGENICITY	-	+	-/+
CELL CYCLE:			
Cyclin D	-	+	-
p21	+	-	+
³H-Thymidine incorporation	-	+	-

Growth Factor Receptors and Integrin Signaling Pathways Are Coordinately Regulated in 3D Structures (Acini)

The ultimate decision a cell makes to proliferate or differentiate is an integrated response to cues derived from both matrix molecules and growth factors within the tissue (5, 7, 54). Although the mechanism by which these extracellular cues are integrated inside the cell is largely unknown, growing evidence suggests that coordinated action of adhesion molecules and growth factors is dependent upon the precise coordination of the intracellular signaling events they induce. We have used the HMT-3522 cell series to explore the intracellular coupling of cell adhesion- and growth factor-dependent signaling and to determine how tissue structure influences signal integration.

EGFR overexpression is commonly associated with breast carcinomas and has emerged recently as a promising target for cancer therapy (55). In comparison with their nontumorigenic counterparts, T4-2 cells display a 10-fold increase in levels of surface expression of EGFR, a transmembrane tyrosine kinase receptor with well-established growth-promoting activity (56). Given the growth-promoting potential of EGFR and its elevated expression in T4-2 tumor cells, it was puzzling to find that inhibition of β1 integrin function in T4-2 cells was sufficient to induce the growth-arrest characteristic of the phenotypic reversion. We therefore asked whether the phenotypic reversion observed upon treatment of 3D cultures with β1 integrin inhibitors might be due to a reciprocal cross-modulation of β1 integrin and EGFR activity: could inhibition of β1 integrin function lead to inhibition of EGFR, and *vice versa*? In support of this hypothesis, we found that inhibition of EGFR function in T4-2 cells (using the function-blocking antibody, monoclonal antibody 225, or the chemical inhibitor, tyrphostin AG1478) resulted in phenotypic reversion that was identical to that observed with β1 integrin inhibitors (not shown; Ref. 41). Each inhibitor, alone, was sufficient to revert the malignant phenotype in this 3D assay.

The observed phenotypic reversion was likely due to the coordinate attenuation of β1 integrin and EGFR function at the level of protein expression and/or at the level of receptor activation. Using Western immunoblot analysis, we found that treatment of T4-2 cell cultures with a β1 integrin inhibitor resulted in the down-modulation of both
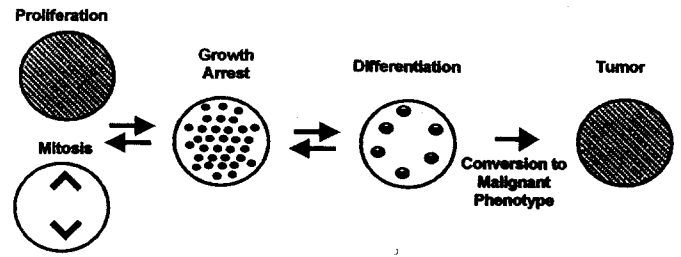

Proliferation

β1 integrin and EGFR protein expression (summarized in Fig. 5); treatment with EGFR inhibitors caused a similar reduction in β1 integrin and EGFR protein levels. In contrast, the levels of E-cadherin were unchanged in S1, T4-2 and reverted T4-2 cells (Ref. 41; not shown). To explore the possibility that coordinate attenuation of receptor function is also achieved at the level of signal activation, β1 integrin and EGFR-specific signaling events were monitored during phenotypic reversion; pp125 FAK phosphorylation and receptor autophosphorylation were used as a measures of β1 integrin (57) and EGFR signal activation (56), respectively. When T4-2 cells in 3D basement membrane cultures were treated with β1 integrin function-blocking antibody, not only did we observe an expected decrease in FAK activity, but we also observed a significant decrease in the phosphorylated or "activated" form of EGFR. Likewise, inhibition of EGFR resulted in the decrease of EGFR autophosphorylation as well as a decrease in FAK activity (41).

Collectively, these studies demonstrate that during phenotypic reversion of tumorigenic T4-2 cells cultured in a 3D basement membrane, protein levels and activity of both β1 integrin and EGFR are restored to levels comparable with those displayed by the nontumorigenic S1 cells. Interestingly, although treatment of 2D T4-2 cell cultures with β1 integrin or EGFR function-blocking agents caused detectable changes in cell behavior and morphology, this treatment did not cause the down-regulation of β1 integrin or EGFR proteins, nor did it result in concomitant attenuation of β1 integrin or EGFR activation (Fig. 5 and data not shown). Thus, the coordinate regulation of β1 integrin and EGFR is dependent upon specific contributions from the 3D basement membrane, presumably in the form of either structural and/or biochemical cues. We interpret these results to mean that normal cells, in a 3D context, use the mechanism of receptor cross-modulation to establish a cellular signaling environment that promotes normal behavior, and that disruption of the mechanisms that regulate these processes can result in tumorigenic behavior.

The ECM-Nuclear Matrix Connection

HMT-3522 cultures in 3D have been used effectively to demonstrate the importance of cell surface receptor coordination in normal and tumorigenic cellular behavior. In addition, this human mammary epithelial cell model has been used also to probe the relationship between nuclear structure and tissue architecture and function. When cultured in 3D basement membrane assays, HMT-3522 S1 cells exhibit a spectrum of cellular behaviors ranging from proliferative to growth arrested to differentiated. Given this behavioral range and the possibility that nuclear architecture itself may impart important behavioral cues (58), we investigated whether these progressive stages of S1 cell differentiation are accompanied by specific nuclear orga-

	3D Cultures				2D Cultures		
	S1	T4-2	T4-2R		T4-2	T4-2R	
Inhibitor Added:	−	−	β1 Integrin Inhibitory Antibody	EGFR Inhibitory Antibody	−	β1 Integrin Inhibitory Antibody	EGFR Inhibitory Antibody
β1 Integrin total levels	+	+++	+	+	+++	+++	+++
EGFR total levels	+	++++	+	+	++++	++++	++++
EGFR* activated	+	++++	+	+	++++	++++	+

Fig. 5. β1 integrin and EGFR protein levels and signal activation are coordinately modulated in HMT-3522 cells cultured in 3D basement membrane assays. When 3D T4-2 cell cultures are treated with functional inhibitors of either β1 integrin or EGFR (*T4-2R*), the cells undergo a phenotypic reversion to give rise to tissue structures that are similar to those displayed by nonmalignant S1 cells. Regardless of the reverting agents used, these treatments result in the coordinate down-modulation of both β1 integrin and EGFR protein levels. Inhibition of β1 integrin or EGFR function also attenuates signal activation of both receptors. Coordinate down-modulation is not observed in cells grown in 2D monolayers (41). These findings demonstrate that adhesion and growth factor receptor activities are coupled in cells cultured in a physiologically relevant context. We propose that in nonmalignant S1 cells, this receptor coupling provides a critical control that dictates the expression and activity of ECM and growth factor receptors, thereby preserving normal cellular behavior.
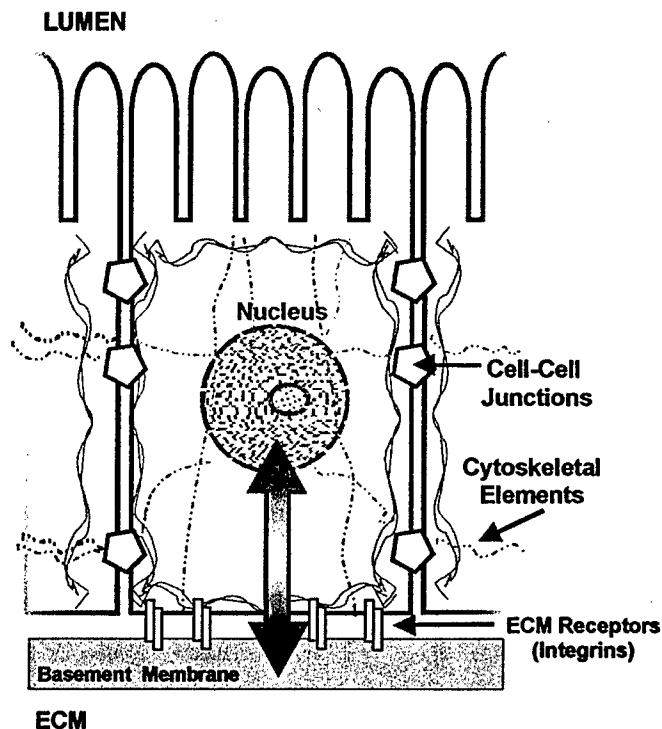
Polarized Mammary Epithelium

LUMEN

Fig. 7. Dynamic reciprocity. Bidirectional flow of tissue-specific information is dependent on the nuclear and chromatin structures, the nature of membrane receptors, and the microenvironmental milieu.

nization patterns. Localization studies performed using probes specific for the NM protein, NuMA, demonstrated that NuMA undergoes dramatic redistribution in S1 cell nuclei during differentiation (Ref. 59; see scheme in Fig. 6). In nuclei of proliferating cells, NuMA protein was diffusely distributed, but as cells arrested growth and underwent morphogenesis into acinar structures, NuMA protein relocalized to distinct subnuclear foci that eventually coalesced into larger aggregates. We predict that breast tumor cells, by virtue of their proliferative capacity, may exhibit a subnuclear organization (*i.e.,* NuMA staining pattern) similar to normal, proliferating hMECs. These studies are in progress. Interestingly, the distribution of the nuclear lamina protein, lamin B, remained unchanged, suggesting that this reorganization is specific for components of the internal NM.

Although these data indicate that NM proteins, such as NuMA, are significantly influenced by extracellular cues provided by the ECM, we suspected that NM architecture itself may also provide important behavioral cues. In support of this, we have demonstrated that treatment of permeabilized S1 acini with a NuMA antibody leads to disruption of the NuMA foci, alteration of histone acetylation, and perturbation of the acinar phenotype (59). These data provide the first evidence for the existence of a dynamic interplay between the ECM, the organization of the nucleus, and the epithelial phenotype. Thus, rather than passively reflecting changes in gene expression, nuclear organization may itself modulate the cellular and tissue phenotype.

Central Hypothesis and Future Directions

The studies summarized above support the hypothesis elaborated from our earlier predictions and set forth again in the abstract (Ref. 3; Fig. 7): the unit of function in higher organisms is neither the genome nor the cell alone, but the tissue itself. The context would determine how individual genes may operate *in vivo*. This concept could explain

why a universal genetic lesion, for example in tumor suppressor genes such as *BRCA-1* (60) and *APC* (61), would give rise to tumors only in specific tissues. A number of other laboratories are modeling tissues other than the mammary gland in 3D context. The combined effort will allow us to decipher how seemingly similar pathways and genes carry out different functions in different tissues. The information generated is complex and intriguing. As such, we will need to develop both new technologies and new connections in various disciplines to unravel the secrets of tissue specificity. To succeed, we will need multi-investigator teams, not only in biology, but also in computation, bioengineering, chemistry, physics and possibly architecture (hence our opening quotation)!

Acknowledgments

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Discussion

Speaker: Would you expect that there is important signaling coming from the cell surface, which I agree with you would be very important, but could it also not be easily disrupted downstream? For example, if there would be double mutations in β-catenin, would that not get the same problem as maybe what you refer to? Lack of interaction?

Dr. Bissell: Of course, but you see, that is the whole point of dynamic reciprocity, in that the signaling goes in both directions. So that you can override the signaling from within and you can override the signaling from outside. But, the point that I am making is that if you are able to restore the structure of the tissue, you then are able to override all of the deletions, mutations, etc. But I assure you that I still absolutely believe in the importance of genes.

Speaker: I'm glad to hear that, but an interesting experiment in that respect would be to, in that particular context of the reversion, to see whether if you would overexpress, for example, β-catenin or the mutant, what will happen?

Dr. Bissell: We, in fact, have done something like this. For example, if we overexpress EGFR, we are able to disrupt normal function. I haven't had time to show you a lot of the studies that we have done; I just had to choose, of course. But, the results are very interesting; if you put EGFR in phenotypically normal tissue, the structure is disrupted. The cells grow disorganized. I am not saying that they necessarily will make a tumor, but under these conditions, now β1 integrin will go up, but it will again go up only in three dimension. If you put the same cells on tissue culture plastic, there will be no detachable increase, despite the fact that EGFR is overexpressed. So, again, the important point here is the difference between how cells regulate themselves, *i.e.,* whether they are in the proper context and in the proper shape, as opposed to being in a monolayer. But, yes, if you overexpress any of the genes involved in these pathways, you will override the structure, even inside a basement membrane, and you will have disorganized growth and possibly tumor at the end.

Second Speaker: I am interested particularly because I did two experiments, one about 10 years ago on lactic acid, and the experiment drew on two different tissue culture plastics. One is primaria, which is a slightly reduced negative charge, and the other is a normal tissue culture plastic. We found not only chromosomal differences but differences in immunohistochemistry, and then when I changed about 5 years ago to OB/GYN cancers, the problem was that we couldn't find more than 75% success in the growth of these cells. So I transferred back to the other plastics, and by doing this we reached 97%, which is published in *Cytogenesis in Ovarian Cancer.* But, now I've started, you have baffled me; I'm confused whether my data are right, because, by culturing the two things, I made two different lots.

Dr. Bissell: I look forward to discussing this with you afterwards, and I think I have some answers from our data that would explain why you got the kind of data you did.

Third Speaker: Do you think the principles you described may be relevant to the development of the so-called liquid tumors, leukemias and lymphomas?

Dr. Bissell: You are raising a very interesting question, because I used to think that blood-borne tumors are an exception to the rule. But as you may know, differentiation of these blood cells, in fact, is very much dependent on the microenvironment in the bone marrow. More recently, people who have used retinoic acid to revert the leukemia or some of these lymphomas, in fact, show that what is affected is the level of $\beta 1$ integrin. Therefore, it is the adhesion pathways that are being affected, so there may be a much wider applicability to this. In addition, there are examples of this in other kinds of epithelial tumors. So I think that if we really look hard enough, while there is a lot of specificity, the broader rules may apply in both blood-borne and solid tumors.

CELL NUCLEUS IN CONTEXT

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ABSTRACT

The molecular pathways that participate in regulation of gene expression are being progressively unraveled. Extracellular signals, including the binding of extracellular matrix and soluble molecules to cell membrane receptors, activate specific signal transducers that convey information inside the cell and can alter gene products. Some of these transducers when translocated to the cell nucleus may bind to transcription complexes and thereby modify the transcriptional activity of specific genes. However, the basic molecules involved in the regulation of gene expression are found in many different cell and tissue types; thus the mechanisms underlying tissue-specific gene expression are still obscure.

In this review we focus on the study of signals that are conveyed to the nucleus. We propose that the way in which extracellular signals are integrated may account for tissue-specific gene expression. We argue that the integration of signals depends on the structural organization of cells (i.e., extracellular matrix, cell membrane, cytoskeleton, nucleus) which defines a particular cell type within a tissue. Putting the nuclei in context allows us to envision gene expression as being regulated not only by the communication between the extracellular environment and the nucleus, but also by the influence of organized assemblies of cells on extracellular-nuclear communications.

Key words: extracellular signal, signal transducer, gene expression, cellular structure, tissue specificity

Segmentation of Nuclei and Cells using Membrane Related Protein Markers

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1997 STANDARD ABSTRACT FORM

NUCLEAR ARCHITECTURE CHANGES AS A FUNCTION OF BOTH CELL GROWTH AND 3-DIMENSIONAL TISSUE ORGANIZATION. ((Sophie A. Lelievre[1], Valerie M. Weaver[1], Carolyn A. Larabell[1], Jeffrey A. Nickerson[2], and Mina J. Bissell[1])). (1) Lawrence Berkeley National Laboratory M.S. 83-101, Berkeley, California 94720; (2) UMass, Dept of Cell Biology, Worcester, MA 01605.

When human mammary epithelial cells are cultivated in a basement membrane, they undergo morphogenesis resembling acini in vivo (Petersen et al., 1992). This process is associated with changes in adhesion molecule organization and the cytoskeleton (Weaver et al., 1997). To investigate if nuclear architecture also undergoes a specific remodeling during morphogenesis, we have studied the localization of a major nuclear structural protein. Immortalized human mammary epithelial HMT-3522 cells are still capable of responding to extracellular matrix (ECM) signals and undergoing morphogenesis analogous to primary cultures of reduction mammoplasty. When cultured in basement membrane-enriched ECM (3-D culture), the cells growth arrest and lay down an endogenous basement membrane. Using immunocytochemistry and confocal microscopy, we find that NuMA, a protein which has been associated with the remodeling of nuclear architecture during mitosis and apoptosis, progressively re-organizes during morphogenesis. NuMA distribution is diffused in proliferating cells (early 2-day cultures) but becomes organized in small foci when the cells progress towards growth arrest (after 4 days of culture). Once the process of morphogenesis is achieved (after 8 to 10 days of culture) NuMA foci become larger and closer to nuclear periphery and will ultimately organize as a peripheral nuclear ring of about 8 large aggregates. When these cells are cultured on plastic (2-D cultures) and induced to growth arrest (less than 5 % cycling cells) by removing epidermal growth factor from the culture medium, NuMA reorganizes into dispersed multifoci, while control proliferating cells show a diffused distribution of the protein. Therefore small dispersed NuMA foci are associated with growth arrest and large peripheral foci are associated with further stages of morphogenesis. The formation of the typical ring of large foci is prevented using various cell treatments which abolish proper tissue architecture. Finally, a 100% co-localization of NuMA and splicing factor B1C8 is observed in the large foci but not in the small foci, suggesting a correlation between the organization of NuMA and elements of the transcriptional machinery during morphogenesis. We are now investigating how such nuclear organization relates to the expression of tissue phenotype.

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THERE IS A $10 FEE TO REPLACE ABSTRACTS

Global rearrangement of the nuclear matrix-associated proteins when human mammary epithelial cells are cultured in 3D: an analysis using confocal-, electron- and soft X-ray microscopy.

Sophie A. Lelièvre, Carolyn A. Larabell, Werner Meyer-Ilse, John T. Brown, Jeffrey A. Nickerson*, Annie Viron†, Edmond Puvion† and Mina J. Bissell. Lawrence Berkeley National Laboratory, Berkeley CA, USA, (*) UMass, Worcester MA, USA, (†) CNRS UPR 9044, Villejuif, France.

Despite the increasing evidence of elaborate nuclear architecture, the existence of "nuclear matrix" (NM) as a true entity of cellular structure is disputed. The argument usually centers on the fact that NM and its associated insoluble proteins have been defined after a series of exhaustive extractions of nuclear components. We are interested in resolving the above dispute: can the structural organization of NM found after high salt extraction be also observed prior to these manipulations? And if so does this organization correlate with different functional states?

Models of 3D culture of cells appear to be ideal for this purpose. Non malignant human mammary epithelial cells grow as monolayer when cultured on plastic, classical 2D system, whereas they form acinus-like differentiated structures when cultured within a reconstituted basement membrane in 3D. Using confocal microscopy, we showed that the distribution of a number of proteins that have been reported to be NM-associated, including Rb, B1C8 and NuMA, was profoundly reorganized between 2D and 3D cultures in both NM preparations and whole, permeabilized cells, and that distribution patterns were distinct for cell proliferation, growth arrest and morphogenesis.

To investigate these structures at a higher resolution, we have utilized soft X-ray microscopy at the Advance Light Source (ALS). This innovative imaging approach permits the examination of intact, hydrated cells at 4-5 times the resolution of light microscopy and discounts the arguments that electron microscopy (EM) images of NM may include artifacts due to the use of critical point-dried samples. Using soft X-ray we showed the presence of a complex filamentous structure associated with nuclear domains not only in NM preparations, confirming results previously obtained in EM, but also in intact cells. Moreover, using immunogold-labeling and silver enhancement, we could tag the nuclear components by soft X-ray microscopy for the first time. The combined use of the three microscopy techniques allowed us to observe changes in the distribution of NuMA, B1C8 and Rb among subcompartments of the nucleus. We believe that parallel application of these tools to 3D systems of culture will improve the understanding between structure and function at the nuclear level. (Supported by BCRP/DOD to SL, France-Berkeley Fund to EP and MJB, and OBER office of DOE to MJB).

Copper Mountain, Co
April 98

Keystone Symposia on
"The nuclear matrix: Involvement in Genomic Organization, function and Cellular Regulation".

Dependence of Nuclear Structure on Tissue Organization During Extracellular Matrix-Induced Mammary Epithelial Cell Morphogenesis.

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We investigated whether the differentiation of epithelial cells into tissue structures imposes a specific nuclear organization. To explore the relationship between nuclear organization and tissue architecture and function, we used an extracellular matrix (ECM)-inducible and dynamic model of human mammary epithelial cell (HMEC) morphogenesis. When cultured within a reconstituted basement membrane (rBM), the HMT-3522 cells form polarized and growth-arrested tissue-like acini with a central lumen, and deposit an endogenous basement membrane (BM). We show here that rBM-induced HMEC morphogenesis is accompanied by the reorganization of the nuclear matrix (NM) proteins NuMA (Nuclear Mitotic Apparatus protein), splicing factor SRm160, and cell cycle regulator Rb. These proteins were found to have distinct distribution patterns specific for proliferation, growth-arrest and acini formation, while the organization of the nuclear lamina protein, lamin B, remained unchanged. In particular, NuMA coalesced progressively into larger foci that were co-localized with large splicing factor speckles as morphogenesis progressed. Moreover, the investigation of NuMA localization in the nuclei of ductal and acinar cells, *in vivo*, on mammary gland tissue section revealed a distribution pattern similar to what was observed in 3D rBM-induced acini. We previously hypothesized (Bissell et al., J. Theor. Biol. 99, 31-68, 1982), and thereafter provided evidence that the ECM directs morphogenesis and gene expression in mammary epithelial cells (Weaver et al., J. Cell Biol. 137, 231-245, 1997; Meyer et al., Mol. Cell. Biol. 18, 2184-2195, 1998). Here we show that a dynamic relationship exists between ECM organization and NM organization during the formation of tissue-like acinar structures. The functional significance of the nuclear organization in mammary epithelial cells will be discussed.

Submitted to Cold Spring Harbor meeting on "dynamic organization of Nuclear function" 7-11 october 98

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Member Society: ASCB

Presentation Preference: Minisymposium OR Poster

Author Conflict of Interest: No

Minisymposium Category Topic Preference: M01 Altering Genomes

Poster Presentation Preference First Choice: E13 Nuclear Matrix and Nuclear Architecture
Poster Presentation Preference Second Choice: C07 Extracellular Matrix & Cell Behavior

Abstract title: Reciprocal interactions between extracellular matrix, nuclear organization and tissue phenotype

Abstract author(s): S Leli$\'{e}$vre1, V Weaver1, J Nickerson2, C Larabell1, A Bhaumik1, O Petersen3 and M Bissell1

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Abstract body: We investigated whether the differentiation of human mammary epithelial cells (HMEC) into functional tissue structures (acini) imposes a specific nuclear organization and if this nuclear organization itself can impose a cellular and tissue phenotype. To explore the relationship between nuclear organization and tissue phenotype, we used an ECM-inducible and dynami model of HMEC morphogenesis. When cultured within a reconstituted basement membrane (rBM), HMT-3522 cells form polarize and growth-arrested tissue-like acini with a central lumen, and deposit an endogenous BM. rBM-induced HMEC morphogenesis was accompanied by the reorganization of the nuclear matrix proteins NuMA, splicing factor SRm160, and cell cycle regulator Rb In particular NuMA coalesced into larger foci as morphogenesis progressed. NuMA was also present in foci, in the human mammary gland in vivo. Perturbation of histone acetylation in the acini altered chromatin organization, disrupted NuMA foci and induced cell proliferation. Moreover, treatment of the permeabilized acini with a NuMA antibody led to the disruption of the NuMA foci, alteration of histone acetylation and perturbation of the acini phenotype. These data provide the first evidence for the existence of a dynamic interaction between the ECM, the organization of the nucleus and the epithelial phenotype. They further show that rather than passively reflecting changes in gene expression, nuclear organization itself can modulate the cellular and tissue phenotype.

NUCLEAR-DIRECTED SIGNALING IN MAMMARY GLAND ACINI.

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Mammary epithelial cells cultured in three-dimensional (3D) matrigel recapitulate the formation of tissue-like structures (acini). Acinar morphogenesis encompasses a proliferation phase followed by growth-arrest, deposition of an endogenous basement membrane and arrangement of cells around a central lumen. We have recently shown that acinar morphogenesis is accompanied by the redistribution of nonchromatin structural proteins (nuclear matrix proteins), including the Nuclear Mitotic Apparatus (NuMA) protein, SR-type of splicing factors, and cell cycle regulators (e.g., Rb) (Lelièvre et al., Proc. Natl. Acad. Sci. 95: 14711-16, 1998). Acinar morphogenesis is also associated with hypoacetylation of histone H4 indicating an alteration in chromatin structure. Trichostatin A (TSA; 41nM, 24hr)-induced hyperacetylation drives acinar cells back into the cell cycle and disrupts acinar organization, respectively shown by increased protein levels of cell cycle and proliferation markers Cyclin D and PCNA and alteration of basement membrane integrity, actin network and NuMA distribution revealed by immunostaining.

To decipher how chromatin structure is involved in growth regulation in acini we have investigated TSA-induced signal transduction. Since our cells are dependent on epidermal growth factor receptor (EGFR) pathway for growth (Wang et al., Proc. Natl. Acad. Sci. 95: 14821-26,1998), we have assessed the level of EGFR and related signaling cascades by western blot analysis following TSA treatment. Both total and activated EGFR and beta1-integrin protein levels were increased. The levels of activated microtubule-associated protein kinase (MAPK), phosphoinositol-3 kinase (Pi3K), and focal adhesion kinase (FAK) were also upregulated. Inhibition of transcription activity using actinomycin D in TSA-treated cells abolished the increase in total/activated EGFR suggesting that histone acetylation may play a role in the transcriptional regulation of EGFR. TSA induced-increase in PCNA protein levels was inhibited by either actinomycin D or tyrphostin, an inhibitor of EGFR pathway, indicating that TSA-induced proliferation is controlled at least partially by EGFR activation. Experiments are underway to unravel the nuclear mechanisms of EGFR activation and define the upstream and downstream events of TSA-induced signaling. The mechanisms by which nuclear organization directs the regulation and maintenance of acinar phenotype will be discussed.

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REPLY TO
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